(54) Title: INHIBITION OF TUMOR METASTASIS VIA NEUTRALIZATION OF TISSUE FACTOR FUNCTION

(57) Abstract

The present invention relates to methods and compositions for preventing, inhibiting, or treating metastasis, as well as to methods of making and using such compositions. More particularly, the present invention relates to molecules that bind tissue factor (TF) and disrupt the TF.VIIa complex, thereby inhibiting the onset and progression of metastasis. In addition, the present invention relates to molecules capable of disrupting tissue factor function, or the function of TF.VIIa complexes. The present invention also relates to methods and compositions useful in disrupting or modulating cell-surface TF expression, including, for example, molecules capable of modifying or disrupting transcription and translation of TF-encoding sequences, or molecules which specifically modulate cellular processing or degradation of TF. The present invention also discloses therapeutic compositions and methods for inhibiting or disrupting tumor-host interactions necessary for initiation and maintenance of the metastatic process.

INHIBITION OF TUMOR METASTASIS
VIA NEUTRALIZATION OF TISSUE FACTOR FUNCTION

5   TECHNICAL FIELD

The present invention relates to methods and
compositions for preventing or treating metastasis.
More particularly, the present invention relates to
molecules that bind tissue factor (TF) and disrupt the
10   TF·VIIa complex, thereby inhibiting the onset and
progression of metastasis. In addition, the present
invention relates to molecules capable of disrupting
tissue factor expression or function, or the function
of TF·VIIa complexes. The present invention also
15   discloses therapeutic compositions and methods for
inhibiting or disrupting tumor-host interactions
necessary for initiation and maintenance of the
metastatic process.

BACKGROUND OF THE INVENTION

20   Metastasis is a multi-step process which requires
highly adapted interactions of tumor cells with host
target organs. The metastatic process has specific
requirements which must be met by both tumor cells and
host factors, in order for the process to be completed
25   successfully.

Hematogenous metastasis leads to disseminated
tumor growth in distant sites and is the final stage in
the progression of many neoplasias. The metastatic
process has been divided into several steps: (1)
30   vascularization of the primary tumor; (2) local
invasion and intravasation of tumor cells into the
vasculature; (3) survival of tumor cells or tumor cell
aggregates in the circulating blood; (4) arrest in the
capillary bed of the target organ; (5) extravasation;
35   and (6) proliferation into the target organ (Fidler, I.

J., _Cancer Res. 50_: 6130-6138 (1990)). Tumor cells can only complete this multi-step process successfully if they have evolved to perform specific functions at each different step and if their specific abilities are

5 complemented by host factors. Clinically and experimentally, different tumors produce metastasis to specific organs, which requires specific contributions from tumor cells and cells in the host organ.

Malignant cells are characterized by their ability

10 to invade locally and their ability to metastasize to distant sites. Tumor cells engage in various highly-adapted interactions with the host to accomplish the multi-step process of metastasis. This involves access of tumor cells to the vasculature by local invasion and

15 entry into the vascular compartment, followed by arrest and implantation of tumor cells in the capillary bed of target organs an subsequent proliferation into metastases (Fidler, _Cancer Res. 50_: 6130-6138 (1990)).

Melanoma metastasis is a paradigmatic model which

20 is useful in the analysis of the molecular pathways of hematogenous metastasis (Clark, _Br. J. Cancer 64_: 631-644 (1991)). Clinically, melanoma metastasizes from the skin to a variety of organs, including the lungs (Akslen, et al, _Invasion Metastasis 8_: 193-204 (1988)).

25 This path of metastasis is recapitulated with many human melanoma cell lines when introduced intravenously into immune deficient mice. (See, eg, Cornil, et al, _J. National Cancer Institute 81_: 938-944 (1989); Mueller, et al, _Cancer Met. Rev. 10_: 193-200 (1992).)

30 Recent studies have identified molecular participants in the metastatic process of tumor cells, such as the expression of adhesion receptors (Chan, et al., _Science 251_: 1600-1602 (1991)), as well as host factors, such as constitutively expressed organ-specific or inducible

35 endothelial cell adhesion molecules (Zhu, et al., _PNAS_

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USA 88: 9568-9572 (1991); Rice, et al., Science 246: 1303-1306 (1989)).

Efficient metastasis of tumor cells has been suggested to be dependent on the plasma coagulation

5   cascades. (See, e.g., Zacharski, et al, Cancer 44: 732-741 (1979); Dvorak, Human Pathol. 18: 275-284 (1987); Murray, Br. J. Cancer 64: 422-424 (1991); Smith, et al, Thromb. Res. 50: 163-170 (1988).) Rather non-selective anticoagulants such as warfarin and

10 heparin can inhibit experimental metastasis of certain tumors in animals (McCulloch, et al, Br. J. Surg. 74: 879-883 (1987); Beuth, et al, Cancer Res. Clin. Oncol. 113: 359-362 (1987)). Studies with the highly-specific thrombin inhibitor hirudin suggest that generation of

15 the pleiotropic serine protease thrombin may trigger essential events during metastasis of melanoma cells (Esumi, et al, Cancer Res. 51: 4549-4556 (1991)).

Other serine proteases of the coagulation system, such as factor VII, have also been suggested as

20 potentially important host factors for metastasis (Francis, et al., Br. J. Cancer 65: 329-334 (1992)). Indeed, factor VII at the site of implantation may bind to tissue factor, its receptor and catalytic cofactor, which can be expressed on host endothelial cells in

25 response to induction by the tumor secreted cytokine vascular permeability factor (Keck, et al., Science 246: 1309-1312 (1989); Clauss, et al., J. Exp. Med. 172: 1535-1545 (1990)). Thus, the formation of an initiating TF·VIIa protease complex on host cells may

30 locally trigger coagulation as an important step for tumor cell implantation and proliferation. However, earlier studies demonstrated expression of TF on a variety of tumor cell lines (Kadish, et al., J. Natl. Cancer Inst. 70: 551-557 (1983); Silberberg, et al.,

35 Cancer Res. 49: 5443-5447 (1989); Bauer, et al.,

Thromb. Res. 56: 425-430 (1989)) indicating that host
cell TF may not be required at the local site of tumor
cell arrest in the vasculature.

TF is a single-chain, 263-amino acid membrane
5   glycoprotein, and its primary sequence indicates
structural similarity with the cytokine receptor family
(Edgington, et al., Thromb. Haemost. 66: 67-79 (1991)).
TF is a transmembrane cell surface receptor, and
functions as the receptor and cofactor for factor VIIa
10   (VIIa) to form a proteolytically active TF·VIIa complex
on cell surfaces (Ruf, et al., J. Biol. Chem. 267:
6375-6381 (1992); Ruf and Edgington, Thromb. Haemos.
66: 529-533 (1991)). The TF extracellular domain binds
factor VIIa and serves as the catalytic cofactor for
15   this serine protease, thereby initiating the plasma
coagulation protease cascades (Edgington, et al.
(1991), supra). Although alternative pathways exist to
activate coagulation, including a tumor cell protease
designated cancer procoagulant (Gordon, et al., Thromb.
20   Res. 6: 127-137 (1975)), the in vivo activation of this
protease system predominantly proceeds via the TF
pathway (Davie, et al., Biochemistry 30: 10363-10370
(1991)).

TF is typically not expressed to any significant
25   degree on cells of the blood or the endothelium, but is
constitutively expressed on certain cell types with a
differentiation-dependent regulation (Drake, et al.,
Am. J. Pathol. 134: 1087-1097 (1989). Promyelocytic
leukemias (Bauer, et al., Thromb. Res. 56: 425-430
30   (1989)) and pancreatic carcinoma cells (Silberberg, et
al., Cancer Res. 49: 5443-5447 (1989)) are examples of
TF expression by non-differentiated precursors of cell
types that normally do not express TF in their fully
differentiated state.
35   Although correlative evidence suggests that

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metastatic tumor cells often express TF, it has not
previously been established whether the expression of
TF is an epiphenomenon or is indeed required for the
metastatic phenotype. Analysis of TF expression on
5   melanoma cells is demonstrated herein to follow the
progression from normal melanocytes towards melanoma
cells with a metastatic phenotype. Furthermore, via
use of a xenograft model, it is disclosed herein that
the selective inhibition of TF on the surface of
10 melanoma cells abolishes prolonged adherence of these
cells in the microvasculature and inhibits pulmonary
metastasis, suggesting a functional importance of TF
expressed on tumor cells rather than on host cells
during the metastatic process.

15   SUMMARY OF THE INVENTION
In contrast to normal melanocytes, malignant
metastasizing human melanoma cells express high levels
of tissue factor (TF), the major cellular initiator of
the plasma coagulation protease cascades. To explore
20 whether TF may contribute to metastatic tumor
dissemination, the effect of specific inhibition of TF
function on human melanoma metastasis in severe
combined immune deficient (scid) mice was analyzed.
Using highly characterized antibodies specific for a
25 functional site on TF, it has now been demonstrated
that initial adherence is insufficient for successful
tumor cell implantation in a target organ. Rapid
arrest of human tumor cells in the lungs of mice was
not diminished by inhibition of TF. However,
30 inhibition of TF receptor function and consequent
reduction in local protease generation abolished
prolonged adherence of tumor cells, resulting in
significantly reduced numbers of tumor cells retained
in the vasculature of the lungs. The growth of
35 pulmonary metastases was also significantly inhibited

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by a blocking anti-TF monoclonal antibody and Fab
fragments thereof, whereas a non-inhibitory antibody
lacked anti-metastatic effects. Cell surface
expression of functional TF thus contributes to
melanoma progression by allowing metastatic cells to
provide requisite signals for prolonged adhesive
interactions and/or transmigration of tumor cells
across the endothelium resulting in successful
metastatic tumor implantation.

A variety of tumor cells have been shown to
express tissue factor (TF) and to initiate the
coagulation serine protease cascades _in vitro_ and _in
vivo_ as evidenced by changes in a variety of markers
for a perturbed hemostatic balance. Presently-
disclosed correlative evidence further indicates an
association of TF with the metastatic phenotype of
tumor cells. However, it is also demonstrated herein
that TF expression and function represents not merely a
correlate of the metastatic phenotype, but a direct
causal role in facilitating metastatic implantation and
growth of tumors.

It is now shown that a specific inhibitor of TF
function, or a specific inhibitor of cell-surface
expression of TF, may be used to block metastasis _in
vivo_. Only human melanoma cell lines with the
metastatic phenotype were found to also express
moderate to high levels of TF. Blocking of tumor cell
TF by binding of an inhibitory monoclonal antibody
(mAb) prevented metastasis, but the binding of an
equally avid and specific non-inhibitory mAb to TF did
not have the same effect. Inhibitory monoclonal
antibodies disclosed herein recognize functional
epitopes on TF, tending to inhibit TF-initiated
coagulation of human plasma by more than 90% and
inhibiting specific binding of factor VII to cultured

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cells expressing TF by more than 80%. The non-inhibitory mAb apparently recognizes a distinct, nonfunctional pitope.

Thus, it seems clear that a function of TF is required for successful implantation of metastatic tumor cells, particularly in view of the presently-disclosed finding that specific interference with TF function, or specific interference with cell-surface expression of TF, profoundly inhibits metastasis.

In addition to monoclonal antibodies to TF, other specific inhibitors of TF function and the TF·VIIa complex also are disclosed to be effective as therapeutic agents useful in the prevention of metastatic spreading of tumors. Targeting tumor cell TF function for therapeutic intervention in the metastatic process is further aided by the lack of TF expression in the vasculature. This would seem to allow efficient and specific intervention without side effects as expected from conventional anticoagulant therapy.

Therefore, in one embodiment, the present invention contemplates a method of preventing or inhibiting metastasis, comprising the administration of a therapeutically effective amount of molecules capable of preventing or inhibiting the expression of human tissue factor (huTF) on the surface of tumor cells. In a preferred embodiment, the molecules are immunoglobulin molecules capable of binding to huTF. In another variation, the invention contemplates a method of preventing or inhibiting metastasis, comprising the administration of a therapeutically effective amount of molecules capable of inhibiting or disrupting huTF function. In one embodiment, the molecules are immunoglobulin molecules. In a preferred embodiment, the immunoglobulin molecules are

antibodies; in other variations, the antibodies are monoclonal or polyclonal.

In various embodiments, monoclonal antibodies may be selected from the group consisting of TF8-5C4, TF8-5G9, TF8-11D12, TF9-1F1, TF9-1D5, TF9-1E7, TF9-1B8, TF9-1B9, TF9-2C4, TF9-2F6, TF9-4D11, TF9-5G4, TF9-5B7, TF9-5C7, TF9-6B4, TF9-6G4, TF9-6C9, TF9-7E10, TF9-8E8, TF9-9E1, TF9-9C3, TF9-9B4, and TF9-10C2. Particularly preferred monoclonal antibodies include TF8-5G9, TF9-6B4, and TF9-6G4. In another variation, methods using monoclonal antibodies capable of immunoreacting with huTF locus I or locus II epitopes, or both, are contemplated.

The invention also contemplates methods utilizing molecules which comprise immunologically active fragments of immunoglobulin molecules. Various embodiments contemplate that the fragments are selected from the group consisting of Fab, Fab', $F(ab)_2$, and F(v) fragments, and CD-grafted humanized mAbs or fragments thereof.

The present invention also contemplates methods and compositions used to inhibit the cell surface expression of TF. In one embodiment, molecules capable of inhibiting cell surface expression of TF are capable of modulating the expression of nucleotide sequences regulating the expression of TF, particularly huTF. In another variation, the molecules are capable of modulating cellular processing mechanisms or expression levels of TF. Methods of identifying useful, cell-surface expression-inhibiting molecules are also disclosed. In one embodiment, the method may comprise the culturing of tumor cells or other TF-expressing cells; admixing the cells with a test compound -- preferably, one that is non-cytotoxic; incubating the resulting admixture for a predetermined period of time;

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and then determining the amount of TF expressed per
cell.

Various preferred embodiments contemplate that
therapeutically-effective molecules and compositions of
5   the present invention may be administered at various
times and dosages, according to the needs of a
particular individual receiving treatment. In one
variation, administration of therapeutically effective
amounts of compounds (e.g., immunoglobulin molecules)
10   according to the present invention begins subsequent to
diagnostic procedures indicating the presence of
tumors, neoplasias, or other malignancies. In other
preferred embodiments, administration of an anti-huTF
antibody or antibody-containing composition as
15   described herein occurs within about 48 hours,
preferably within about 12-36 hours, more preferably
within about 2-8 hours and most preferably immediately
prior to or substantially concurrently with invasive
surgical procedures directed at diagnosis or removal of
20   a tumor or malignancy.

In one variation, the molecules are administered
within about 1-8 hours of invasive surgical procedures
directed at diagnosis or removal of a tumor or
malignancy. In another embodiment, the molecules are
25   administered substantially concurrently with invasive
surgical procedures directed at diagnosis or removal of
a tumor or malignancy. In yet another variation, the
molecules are administered as a single bolus 30 minutes
prior to surgery. In one embodiment, the bolus
30   comprises a dose of about 500µg/kg.

The invention further contemplates administration
of therapeutically effective amounts of preferred
molecules, wherein preferred amounts for administration
are in the range of about 0.05 to 20 milligrams per
35   kilogram body weight per day (mg/kg/day). More

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preferably, the administered dose is in the range of
0.1 to 10mg/kg/day. In one variation, the molecules
are administered intravenously. Other routes/modes of
administration are also contemplated. In various
5   preferred embodiments, the therapeutically effective
amount is an amount sufficient to produce plasma levels
of the effective ingredient (e.g., antibody molecules)
of about 0.05µg/ml to about 25µg/ml; in another
embodiment, an effective amount is in the range of
10   about 0.1µg/ml to about 10µg/ml. In another
embodiment, the therapeutically effective amount is an
amount sufficient to produce plasma levels of the
effective ingredient of about 1µg/ml to about 5µg/ml.
     Therapeutically effective amounts of the
15   presently-disclosed molecules and compositions
administrable according to the within-disclosed methods
also include amounts sufficient to inhibit, and
preferably inhibit by at least about 10-20 percent,
more preferably 30 percent, even more preferably by at
20   least about 50 percent, or about 70 percent, and most
preferably by at least about 90 percent, the cell
surface expression of TF, or its function, on
potentially metastatic cells. In another embodiment, a
therapeutically effective amount is one sufficient to
25   inhibit binding of huTF to factor VII or VIIa by at
least about 40%, more preferably by about 60%, and even
more preferably by at least about 80%. The present
invention also contemplates the administration of
therapeutically effective amounts of the presently-
30   disclosed compounds sufficient to produce similar
degrees or percentages of inhibition of a clinically
significant increase in the surface expression of TF on
potentially metastatic cells, e.g., tumor cells.
     In another aspect, the present invention
35   contemplates methods using molecules capable of

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inhibiting the binding of factor VII or VIIa to huTF.
In one embodiment, the molecules are capable of
disrupting huTF·VIIa complex function. In another
variation, the molecules are capable of preventing or
5   inhibiting the activation of substrates and the
coagulation cascade via binding to TF·VIIa complex.

The present invention also contemplates methods of
identifying molecules capable of inhibiting metastasis.
In one such method, cells derived from metastatic
10   tissue -- or from another TF-producing source -- are
isolated and suspended; the suspension is then
incubated with a sample of the molecules for a
predetermined period of time, to form an admixture; and
the admixture is subsequently assayed for TF specific
15   activity, thereby assessing the ability of the
molecules to inhibit metastasis. An assay according to
the present invention may comprise (a) incubating the
admixture with a TF-specific antibody-containing
solution for a predetermined period of time; (b)
20   incubating the second admixture of (a) with a labeled
immunoglobulin or a labeled immunoglobulin fragment for
a predetermined period of time; and (c) assaying for
the presence of labeled TF-antibody complexes. In
various embodiments, the source of TF may be tumor
25   cells or other potentially-metastatic tissues, or the
source may be normal cells or tissues, which may be
processed to achieve various degrees of purity.

In another embodiment, the present invention
contemplates a method for identifying compounds or
30   molecules capable of inhibiting, suppressing or
preventing the expression of human tissue factor (huTF)
on the surface of tumor cells. In one variation, such
a method comprises culturing tumor cells; admixing the
cells with a compound to be tested -- preferably, a
35   compound which does not affect the viability of the

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cells; incubating the admixture for a predetermined period of time; and assaying for or determining the amount of TF expressed per cell.

The present invention also contemplates various

5 therapeutic compositions. In one embodiment, a therapeutic composition comprises, in unit dose form, molecules capable of preventing or inhibiting the expression of huTF on the surface of tumor cells, in a pharmaceutically acceptable excipient. In a preferred

10 embodiment, the molecules are immunoglobulin molecules and are capable of binding to huTF. In another variation, a therapeutic composition of the present invention comprises, in unit dose form, molecules capable of inhibiting or disrupting huTF function, in a

15 pharmaceutically acceptable excipient.

In various aspects of the invention, the molecules may comprise immunoglobulin molecules or immunologically active fragments of immunoglobulin molecules. In alternative embodiments, the molecules

20 comprise anti-huTF antibody molecules, such as polyclonal or monoclonal antibodies, including, for example, monoclonal antibodies selected from the group consisting of TF8-5C4, TF8-5G9, TF8-11D12, TF9-1F1, TF9-1D5, TF9-1E7, TF9-1B8, TF9-1B9, TF9-2C4, TF9-2F6,

25 TF9-4D11, TF9-5G4, TF9-5B7, TF9-5C7, TF9-6B4, TF9-6G4, TF9-6C9, TF9-7E10, TF9-8E8, TF9-9E1, TF9-9C3, TF9-9B4, and TF9-10C2. In another variation, monoclonal antibodies TF8-5G9, TF9-6B4, and TF9-6G4 are particularly preferred. In other variations, the

30 molecules are capable of inhibiting the binding of factor VII or VIIa to huTF, or they are capable of disrupting huTF·VIIa complex function.

Finally, the present invention also contemplates methods of inhibiting huTF synthesis in the vasculature

35 using anti-sense oligonucleotides, as well as methods

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and compositions for inhibiting the catalytic site of factor VIIa in the huTF·VIIa complex. Further contemplated is the use of peptidyl and other pharmaceutical compositions to interfere with huTF ↔

5   VII/VIIa interactions. Disruption or inhibition of the TF pathway using compounds, compositions and methods of the present invention is also contemplated.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates TF expression by cultured
10   human melanocytes and melanoma cell lines. The various cell lines used are shown on the vertical axis; procoagulant activity expressed in $mU/10^6$ cells is shown on the horizontal axis. TF specific activity per cell line is also illustrated. TF functional activity
15   was analyzed in a plasma coagulation assay after octyl-glucopyranoside lysis of cell pellets of normal human epidermal melanocytes (NHEM) and melanoma cell lines WM35, M24met, and C8161. TF specific activity was assessed after blocking TF in cell lysate by a 30
20   minute preincubation with mAb TF8-5G9 (30µg/ml). Means from four independent experiments are illustrated.

Figures 2A and B illustrate the surface expression of TF on metastatic melanoma cell lines. In each instance, the number of cells is plotted on the
25   vertical axis, while fluorescence intensity is shown on the horizontal axes. Indirect immunofluorescence and flow cytometry were used to demonstrate TF antigen on the surface of M24met (Fig. 2A) and C8161 (Fig. 2B) melanoma cell lines. Cells were incubated with 50µg/ml
30   isotype matched control Ab (TIB115; solid line), or anti-TF mAb TF8-5G9 (bold line) and TF9-10H10 (dotted line), respectively. Bound mAb was visualized by a secondary goat anti-mouse IgG conjugated to fluorescein isothiocyanate. Results of a representative experiment
35   are shown in each of 2A and 2B.

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Figure 3A illustrates functional activity of human
TF in human and mouse plasma. Clotting time in seconds
is plotted on the vertical axis, whereas the amount of
human TF (in ng/ml) is shown on the horizontal axis.

5   The ability of phospholipid reconstituted recombinant
human TF to initiate coagulation in mouse plasma (open
circles) was evaluated in a standard clotting assay in
comparison to pooled normal human plasma (closed
circles). Figure 3B shows that, in either case, a

10   preincubation with 30µg/ml TF8-5G9 -- but not with TF9-
10H10 -- inhibited TF-induced coagulation. In Fig. 3B,
procoagulant activity (mU/ml) is shown on the vertical
axis. On the horizontal axis, results of preincubation
of human vs. mouse plasma with 30µg/ml control

15   (TIB115), 10H10 (TF9-10H10) or 5G9 (TF8-5G9) mAb is
shown.

Figures 4A and 4B illustrate the distribution of
radiolabeled M24met cells after intravenous injection
into scid mice. On the vertical axis, time in minutes

20   (4A) or hours (4B) is shown. On the horizontal axis,
the percent of injected dose is illustrated, in liver,
blood, and lungs. In 4A, $p = 0.018$; in 4B, $p = 0.005$.
Cells were labeled with 5-(^{125}I)-iodo-2'-deoxyuridine
for 24 hours, washed and incubated for 45 minutes with

25   1mg monoclonal antibody TF8-5G9 (white bars) or buffer
control (dark bars). Radiolabeled M24met cells were
injected into the tail vein of scid mice. Mice were
bled and sacrificed at different time points, as shown.
Means and standard deviations are based on four animals

30   per group at each time interval. The probability of no
difference occurring between groups at a given time was
assessed with the Wilcoxon rank sum test.

Figure 5 illustrates the ability of anti-TF
monoclonal antibodies to inhibit tumor associated TF in

35   vivo and the TF expression of M24met melanoma cells in

vivo. Tissue factor procoagulant activity in mU/mg is
indicated on the vertical axis, whereas TF expression
in the presence or absence of mAb TF8-5G9 in cultured
cells, subcutaneous tumors, and lymph node metastasis

5   is represented by the dark bars (- TF8-5G9) and light
bars (+ TF8-5G9) extending vertically from the
horizontal axis.

DETAILED DESCRIPTION

A.   Definitions

10   "Amino acid": The amino acid residues described
herein are preferred to be in the natural L-
configuration. However, residues in the "D" isomeric
form can be substituted for any L-amino acid residue,
as long as the desired functional property of

15   immunoglobulin binding is retained by the polypeptide.
NH_2 refers to the free amino group present at the amino
terminus of a polypeptide, while COOH refers to the
free carboxy group present at the carboxy terminus of a
polypeptide. In keeping with standard polypeptide

20   nomenclature, J. Biol. Chem. 243: 3557-59, (1969),
abbreviations for amino acid residues are as shown in
the following Table of Correspondence:

TABLE OF CORRESPONDENCE

SYMBOL		AMINO ACID
1-Letter	3-Letter	
Y	Tyr	L-tyrosine
G	Gly	glycine
F	Phe	L-phenylalanine
M	Met	L-methionine
A	Ala	L-alanine
S	Ser	L-serine
I	Ile	L-isoleucine
L	Leu	L-leucine
T	Thr	L-threonine

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V	Val	L-valine
P	Pro	L-proline
K	Lys	L-lysine
H	His	L-histidine
Q	Gln	L-glutamine
E	Glu	L-glutamic acid
W	Trp	L-tryptophan
R	Arg	L-arginine
D	Asp	L-aspartic acid
N	Asn	L-asparagine
C	Cys	L-cysteine

It should be noted that all amino acid residue sequences are represented herein by formulae whose left to right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a bond to a further sequence of one or more amino acid residues up to a total of about fifty residues in the polypeptide chain.

The term "antibody" in its various grammatical forms is used herein to refer to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, i.e., molecules that contain an antibody combining site or paratope. Illustrative antibody molecules are intact immunoglobulin molecules, substantially intact immunoglobulin molecules and those portions of an immunoglobulin molecule that contain the paratope, including those portions known in the art as Fab, Fab', F(ab')$_2$ and F(v).

Fab and F(ab')$_2$ portions of antibody molecules may be prepared by the proteolytic reaction of papain and pepsin, respectively, on substantially intact antibody molecules by methods that are well known. (See, e.g.,

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U.S. Pat. No. 4,342,566 to Theofilopoulos, et al., the
disclosures of which are hereby incorporated by
reference.)   Fab' antibody molecule portions are also
well known and are produced from F(ab')$_2$ portions
5   followed by reduction of the disulfide bonds linking
the two heavy chain portions as with mercaptoethanol,
and followed by alkylation of the resulting protein
mercaptan with a reagent such as iodoacetamide.   An
antibody containing intact antibody molecules is
10   preferred, and is utilized as illustrative herein.

The term "antibody combining site" refers to that
structural portion of an antibody molecule comprised of
a heavy and light chain variable and hypervariable
regions that specifically binds (immunoreacts with)
15   antigen.

The word "antigen" has been used historically to
designate an entity that is bound by an antibody, and
also to designate the entity that induces the
production of the antibody.   More current usage limits
20   the meaning of antigen to that entity bound by an
antibody, while the word "immunogen" is used for the
entity that induces antibody production.

In some instances, the antigen and immunogen are
the same entity as where a synthetic polypeptide is
25   utilized to induce production of antibodies that bind
to the polypeptide.   However, the same polypeptides may
also be able to induce antibodies that bind to a whole
protein, in which case the polypeptide is both
immunogen and antigen, while the whole protein is an
30   antigen.   Where an entity discussed herein is both
immunogenic and antigenic, it will generally be termed
an antigen.

The term "antigenic determinant" refers to the
actual structural portion of the antigen that is
35   immunologically bound by an antibody combining site.

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The term is also used interchangeably with "epitope".

The term "immunoreact" in its various forms is used herein to refer to binding between an antigenic determinant-containing molecule and a molecule
5   containing an antibody combining site such as a whole antibody molecule or a portion thereof.

As used herein, the term "specifically bound" refers to a non-random binding reaction between a cell surface receptor and a ligand molecule.
10   The phrase "monoclonal antibody" in its various grammatical forms refers to an antibody containing or having only one species of antibody combining site capable of immunoreacting with a particular antigen. A monoclonal antibody thus typically displays a single
15   binding affinity for any antigen with which it immunoreacts. A monoclonal antibody may therefore contain an antibody molecule having a plurality of antibody combining sites, each immunospecific for a different antigen, e.g., a bispecific (chimeric)
20   monoclonal antibody.

The phrase "monoclonal antibody composition" in its various grammatical forms refers to a population of antibody molecules that contain only one species of antibody combining site capable of immunoreacting with
25   a particular antigen. A monoclonal antibody composition thus typically displays a single binding affinity for any antigen with which it immunoreacts. A monoclonal antibody composition may therefore contain an antibody molecule having a plurality of antibody
30   combining sites, each immunospecific for a different antigen, e.g., a bispecific monoclonal antibody.

A monoclonal antibody composition is typically composed of antibodies produced by clones of a single cell called a hybridoma that secretes (produces) but
35   one kind of antibody molecule. The hybridoma cell is

formed by fusing an antibody-producing cell and a
myeloma or other self-perpetuating cell line. Such
antibodies were first described by Kohler and Milstein,
Nature 256: 495-497 (1975), which description is

5   incorporated by reference herein.

        A monoclonal antibody composition can also be
produced by methods well known to those skilled in the
art of producing chimeric antibodies. Those methods
include isolating, manipulating, and expressing the

10   nucleic acid that codes for all or part of an
immunoglobulin variable region including both the
portion of the variable region comprised by the
variable region of immunoglobulin light chain and the
portion of the variable region comprised by the

15   variable region of immunoglobulin heavy chain. Methods
for isolating, manipulating, and expressing the
variable region coding nucleic acid in procaryotic and
eucaryotic hosts are disclosed in Robinson et al., PCT
Publication No. WO 89/0099; Winter et al., European

20   Patent Publication No. 0239400; Reading, U.S. Patent
No. 4,714,681; Cabilly et al., European Patent
Publication No. 0125023; Sorge et al., Mol. Cell Biol.
4: 1730-1737 (1984); Beher et al., Science 240: 1041-
1043 (1988); Skerra et al., Science 240: 1030-1041

25   (1988); and Orlandi et al., PNAS USA 86: 3833-3837
(1989). (The disclosures of the art cited in the
present disclosure are hereby incorporated by reference
herein.)

        Typically, the nucleic acid codes for all or part

30   of an immunoglobulin variable region that binds a
preselected antigen (ligand). Sources of such nucleic
acids are well known to one skilled in the art and, for
example, may be obtained from a hybridoma producing a
monoclonal antibody that binds the preselected antigen,

35   or the preselected antigen may be used to screen an

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expression library coding for a plurality of immunoglobulin variable regions, thus isolating the nucleic acid.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and their grammatical variations, as they refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a mammal, particularly a human, without the production of allergic or untoward physiological effects such as nausea, dizziness, gastric upset and the like.

The phrase "substantially simultaneously" or "substantially concurrently" is used herein to mean within a time period sufficient to produce concurrent results, e.g., prevention or inhibition of the spread of malignant cells -- i.e., metastasis -- that may occur as a result of an invasive surgical procedure by administration of an anti-huTF antibody, anti-huTF antibody-containing composition, or a subcombination or combination thereof, as described herein.

Also, as used herein, "pg" means picogram, "ng" means nanogram, "μg" means microgram, "mg" means milligram, "kg" means kilogram, "μl" means microliter, "ml" means milliliter, and "l" means liter.

B.   Therapeutic Methods and Compositions

1.   Methods

The present invention contemplates methods of treating and/or preventing metastasis, and compositions useful therefor. Patients in need of such treatment include those at risk for or suffering from metastatic or potentially-metastatic tumors, malignancies, or neoplasias, such as melanoma and the like. Patients particularly able to benefit from the present invention are those with diagnosed tumors, neoplasias, or other

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malignancies, particularly those with a tendency to
metastasize.

Thus, in one embodiment, the present invention
contemplates a method of preventing or inhibiting

5   metastasis by administering to a patient in need of
such treatment a therapeutically effective amount of a
molecule that interferes with cell-surface expression
of TF or with TF function. Methods of interfering with
the cell-surface expression of TF may preferentially

10 utilize molecules or compounds capable of interfering
with or modulating the expression of TF-related genes,
for example. In general, molecules capable of
disrupting cell-surface expression of TF must be of
sufficiently small size to facilitate the entry of such

15 molecules into the cell. Also, such molecules are
preferably non-toxic to the cells. Molecules capable
of disrupting or modulating cell-surface TF expression
may include, for example, nucleotide sequences encoding
regulatory molecules, or the regulatory molecules

20 themselves. Other examples of molecules which may
suppress cell-surface expression of TF include
molecules which specifically modulate cellular
processing and degradation of TF.

Methods of interfering with TF function may

25 preferentially employ immunoglobulins, such as anti-TF
antibodies. A more preferred method utilizes an anti-
huTF antibody. An even more preferred method utilizes
an inhibitory anti-huTF antibody specific for locus I
or II epitopes -- or both -- as described in Ruf, et

30 al., Biochem. J. 278: 729-733 (1991), which is
incorporated herein by reference. Essentially,
antibodies to the locus I epitope include inhibitory
(e.g., TF8-5G9) and non-inhibitory (e.g., TF9-10H10)
antibodies. Antibodies to the locus II epitope include

35 inhibitory (e.g. TF9-6B4, TF9-6G4) and non-inhibitory

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antibodies as well. The locus I epitope appears to be
encoded by exons 4 and 5 of huTF, whereas the locus II
epitope appears to be encoded by exon 3 of huTF.
Applicants do not intend, however, for the present

5   invention to be limited by a particular hypothesis or
theory of operation.

       Exemplary antibody molecules include intact
immunoglobulin molecules, substantially intact
immunoglobulin molecules, and those portions of an

10   immunoglobulin molecule that contain the paratope,
including those portions known in the art as Fab, Fab',
$F(ab)_2$ and F(v).

       The phrase "therapeutically effective amount" is
used herein to mean an amount sufficient to inhibit,

15   and preferably inhibit by at least about 10 percent,
more preferably by about 20-30 percent, even more
preferably by at least about 50 percent, or about 70
percent, and most preferably by at least 90 percent, a
clinically significant increase in the surface

20   expression of TF on potentially metastatic cells, e.g.,
tumor cells. Preferred therapeutically effective
amounts for the agents used herein as active
ingredients are provided below. A clinically
significant increase in the cell surface expression of

25   TF on tumor cells is an increase of about 10 times the
normal level to about 50,000 times the normal level.
Often, a clinically significant increase is an increase
of about 100 to 1,000 times the normal level of
surface-expressed huTF. Methods for determining the

30   level of TF on cell surfaces are well known in the art;
particularly preferred methods are those described
herein, in Section H. A therapeutically effective
amount of the agents used herein may also comprise an
amount sufficient to inhibit about 1-20 percent, and

35   preferably inhibit by about 20-50 percent, more

preferably by about 40-70 percent, even more preferably
by about 80-100 percent, the functional activity of
huTF. Methods of assessing the functional activity of
TF are known in the art; a preferred method is that
5   described hereinbelow in Section G.

In another embodiment, the method comprises
administering to a patient in need of treatment (i.e.,
with metastatic tumors or at risk for metastasis) a
therapeutically effective amount of an anti-human TF
10   (huTF) antibody, preferably an amount sufficient to
inhibit huTF functional activity _in vivo_. In yet
another variation, the method comprises administering
to a patient in need of treatment a therapeutically
effective amount of a molecule capable of inhibiting or
15   preventing the cell-surface expression of huTF.

In another embodiment, a therapeutic method of the
present invention comprises administering a
therapeutically effective amount of an anti-human TF
antibody, preferably an affinity-purified polyclonal
20   antibody, and more preferably a mAb. In addition, the
anti-huTF antibody molecules used herein may be in the
form of Fab, Fab', $F(ab')_2$ or F(v) portions of whole
antibody molecules. Preferably, the amount of anti-
huTF antibody administered is sufficient to reduce by
25   at least about 20 percent, preferably by at least 40
percent, and more preferably by at least 60 or 80
percent, a clinically significant increase in the
levels of tumor cell surface-expressed, functional huTF
in a patient diagnosed as having tumors, malignancies,
30   or neoplasia, or displaying at least one of the
symptoms associated therewith. As previously
discussed, patients capable of benefiting from this
method include those suffering from malignancies in
which metastasis is not uncommon.
35   Methods for isolating TF and for inducing anti-

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huTF antibodies are well known in the art. (See, for
example, U.S. Patent No. 5,110,730 to Edgington, et
al.) Various methods for determining and optimizing
antibody affinities and inhibitory abilities are also
5 known in the art. Preferred methods of determining and
optimizing the ability of an anti-huTF antibody to
inhibit the binding of huTF or the formation of TF·VIIa
complexes on cell surfaces and thereby inhibit TF-
induced metastasis are discussed in Section G and in
10 the Examples hereinbelow.

2. Compositions

Useful compositions disclosed herein include, as
an active ingredient, molecules capable of inhibiting
or preventing the cell-surface expression of huTF.
15 Molecules capable of disrupting or modulating cell-
surface TF expression may include, for example,
nucleotide sequences encoding regulatory molecules, or
the regulatory molecules themselves. Other examples of
molecules which may suppress cell-surface expression of
20 TF include molecules which specifically modulate
cellular processing and degradation of TF.

Compositions of the present invention also include
those in which the active ingredient is capable of
inhibiting TF function. Preferred active ingredients
25 according to the present invention include, for
example, immunoglobulins and fragments thereof,
including antibodies and antibody fragments.

Preferably, an anti-huTF antibody used in a
therapeutic method of this invention is an affinity
30 purified polyclonal antibody. More preferably, the
antibody is a monoclonal antibody (mAb). In addition,
preferred anti-huTF antibody molecules used herein may
be in the form of Fab, Fab', $F(ab')_2$ or F(v) portions
of whole antibody molecules.

35 Preferred monoclonal antibodies useful in

practicing the present invention are those capable of
being produced by a hybridoma such as hybridoma TF8-5G9
or TF9-6B4, described in U.S. Pat. No. 5,110,730 to
Edgington, et al. While mAbs such as TF8-5G9 and TF9-
6B4 can be produced by hybridoma culture, the invention
is not so limited. Also contemplated is the use of
mAbs produced by an anti-TF immunoglobulin expressing
nucleic acid cloned from a hybridoma such as TF8-5G9,
TF9-6B4, or TF9-6G4. That is, the nucleic acid
expressing the anti-TF antibody molecules secreted by
hybridomas TF8-5G9, TF9-6B4, or TF9-6G4 and the like
can be transferred into another cell line to produce a
transformant. The transformant is genotypically
distinct from the original hybridoma but is also
capable of producing anti-huTF antibody molecules,
including immunologically active fragments of whole
antibody molecules, corresponding to those secreted by
the hybridoma. See, for example, United States Patent
No. 4,642,334 to Reading; PCT Publication No. WO 890099
to Robinson et al.; European Patent Publications No.
0239400 to Winter et al. and No. 0125023 to Cabilly et
al.

Preferred antibodies or fragments thereof display
an immunoreactivity for huTF that is similar to that of
those produced by the above-described hybridomas. As
used herein, the term "immunoreactivity" in its various
grammatical forms refers to the concentration of
antigen required to achieve a 50% inhibition of the
immunoreaction between a given amount of the antibody
and a given amount of huTF antigen. That is,
immunoreactivity is the concentration of antigen
required to achieve a B/B_0 value of 0.5, where B_0 is
the maximum amount of antibody bound in the absence of
competing antigen and B is the amount of antibody bound
in the presence of competing antigen, and both B_0 and B

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have been adjusted for background.   (See, e.g., Robard, Clin. Chem. 20: 1255-1270 (1974).)

   Preferred anti-huTF antibodies according to the present invention also include antibodies that inhibit
5 or interfere with huTF function.   Such preferred antibodies include antibodies to huTF which are capable of inhibiting the binding of factor VII or VIIa.   More preferred are huTF antibodies which are specific for primates, and more preferably humans (i.e., such
10 antibodies do not cross-react with TF from non-primates, and more preferably, do not cross-react with TF from non-human primates).   Exemplary preferred monoclonal antibodies are listed in Table 1 below.

Table 1
Monoclonal Antibodies to huTF*

	Name	Isotype	% Inhibition of: Coag.[a]	VII[b]	Inhib. of primate TF
5	TF8-5C4	IgG$_1$, kappa	96	57	--
	TF8-5G9	IgG$_1$, kappa	99	80	--
	TF8-11D12	IgG$_1$, kappa	99	82	--
	TF9-1F1	IgG$_1$, kappa	95	83	M,B[c]
	TF9-1D5	IgG$_1$, kappa	95	76	M,B
10	TF9-1E7	IgG$_1$, kappa	97	90	M,B
	TF9-1B8	IgG$_1$, kappa	98	83	M,B
	TF9-1B9	IgG$_1$, kappa	97	84	M,B
	TF9-2C4	IgG$_1$, kappa	97	78	M,B
	TF9-2F6	IgG$_1$, kappa	97	79	M,B
15	TF9-4D11	IgG$_1$, kappa	97	81	M,B
	TF9-5G4	IgG$_1$, kappa	97	83	M,B
	TF9-5B7	IgG$_1$, kappa	97	74	M,B
	TF9-5C7	IgG$_1$, kappa	96	72	M,B
	TF9-6B4	IgG$_1$, kappa	96	98	M,B
20	TF9-6G4	IgG$_1$, kappa	95	78	M,B
	TF9-6C9	IgG$_1$, kappa	95	47	--
	TF9-7E10	IgG$_1$, kappa	97	54	--
	TF9-8E8	IgG$_1$, kappa	97	76	M,B
	TF9-9E1	IgG$_1$, kappa	90	71	M,B
25	TF9-9C3	IgG$_1$, kappa	97	82	M,B
	TF9-9B4	IgG$_1$, kappa	95	82	M,B
	TF9-10C2	IgG$_1$, kappa	98	71	M,B
	TF9-10H10	IgG$_1$, kappa	0	20	--

30   [a]   Inhibition of coagulation of human plasma
            induced by purified human brain TF.

      [b]   Inhibition of specific ^{125}I-factor VII binding
            to J82 cells.

      [c]   Inhibition of coagulation of human plasma

35   induced by B (crude baboon brain extract) or
            M (lysed COS cells -- African green monkey

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kidney cells). A letter is entered for a
species if the antibody inhibited the
procoagulant activity by $\geq$ 60%.

*   (Adapted from Morrissey, et al., _Thromb. Res._
5   _52_: 247-261 (1988).)

_____

Other preferred anti-huTF antibodies useful in
practicing the present invention may also
10   immunologically cross-react with a peptide analog of
TF, and more preferably, an analog of huTF. A "TF
peptide analog" is a polypeptide capable of
competitively inhibiting the binding of factor VII or
VIIa to TF expressed on the surface of tumor cells.
15   Preferred TF peptide analogs include the epitopes
identified as locus I or locus II, or both. Other
preferred anti-huTF antibodies useful in practicing the
present invention immunologically cross-react with
recombinant TF or with TF mutants which are
20   functionally similar to, or include the same functional
epitope as, native huTF. Such mutant TFs include,
without limitation, TF$_{A165A166}$ (Ruf, et al., _J. Biol._
Chem. 267: 6375-6381 (1992)).

Monoclonal antibody compositions are also
25   contemplated by the present invention. A monoclonal
antibody composition contains, within detectable
limits, only one species of antibody combining site
capable of effectively binding huTF. Thus, a
monoclonal antibody composition of the present
30   invention typically displays a single binding affinity
for huTF, even though it may contain antibodies capable
of binding proteins other than huTF. In one
embodiment, a monoclonal antibody composition contains
antibodies capable of inhibiting the function of huTF.
35   In another embodiment, a monoclonal antibody

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composition contains antibody molecules that
immunoreact with huTF and a polypeptide analog of the
tissue factor epitope located near the carboxy terminus
of huTF, preferably p106-219, and more preferably, the
5   locus I epitope. In another preferred embodiment, the
antibody molecules immunoreact with huTF and a
polypeptide analog of the tissue factor epitope
identified as the locus II epitope. The antibody
molecules contained in these compositions immunoreact
10   with huTF at or near its locus I or II epitopes and
thereby neutralize its functionality and/or its ability
to bind factor VII or VIIa.

     A monoclonal antibody composition of the present
invention can be produced by initiating a monoclonal
15   hybridoma culture comprising a nutrient medium
containing a hybridoma that secretes antibody molecules
of the appropriate polypeptide specificity. The
culture is maintained under conditions and for a time
period sufficient for the hybridoma to secrete the
20   antibody molecules into the medium. The antibody-
containing medium is then collected. The antibody
molecules can then be further isolated by well-known
techniques.

     Many techniques are also available for generating
25   antibodies to specific cell surface receptors; such
antibodies may be useful when administered in
conjunction with, or conjugated to, anti-TF antibodies
of the present invention. Examples of known methods of
generating antibodies to cell surface receptors include
30   U.S. Patent No. 4,816,567 and published PCT application
no. WO90/14424. Various methods of targeting specific
cells using antibodies are also known; see, e.g.,
Huang, et al., J. Biol. Chem. 255: 8015-81 (1980).
(References cited herein are incorporated by
35   reference.)

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Media useful in the preparation of these
compositions are both well-known in the art and
commercially available, and include synthetic culture
media, inbred strains of mice, and the like. An
5   exemplary synthetic medium is Dulbecco's minimal
essential medium (DMEM; Dulbecco, et al., Virol. 8: 396
(1959)) supplemented with 4.5g/l glucose, 20mM
glutamine, and 20% fetal calf serum. An exemplary
inbred mouse strain is the BALB/c strain. The
10   monoclonal antibody compositions produced by the above
methods can be used, for example, in diagnostic and
therapeutic modalities wherein formation of an huTF-
containing immunoreaction product is desired.

The preparation of therapeutic compositions which
15   contain polypeptides, antibody molecules, or portions
thereof as active ingredients is well understood in the
art. Typically, such compositions are prepared as
injectables, either as liquid solutions or suspensions;
however, solid forms suitable for solution in, or
20   suspension in, liquid prior to injection can also be
prepared. The preparation can also be emulsified. The
active therapeutic ingredient is often mixed with
excipients which are pharmaceutically acceptable and
compatible with the active ingredient. Suitable
25   excipients include, for example, water, saline,
dextrose, glycerol, ethanol, or the like, and
combinations thereof. In addition, if desired, the
composition can contain minor amounts of auxiliary
substances such as wetting or emulsifying agents, or pH
30   buffering agents which enhance the effectiveness of the
active ingredient.

A therapeutic composition of the present invention
may also take the form of tablets, pills, capsules,
sustained release formulations, or powders. In any
35   case, the compositions preferably contain from about

10%-95% active ingredient, and more preferably, about 25%-70% active ingredient.

A polypeptide or antibody can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The therapeutic polypeptide- or antibody-containing compositions are conventionally administered intravenously, as by injection of a unit dose, for example. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined quantity of active material calculated to produce the desired therapeutic effect in association with the required diluent; i.e.., carrier, or vehicle.

The compositions are administered in a manner compatible with the dosage formulation, and in a therapeutically effective amount. The quantity of the effective ingredient to be administered depends on the subject to be treated, capacity of the subject's immune system to utilize the active ingredient, and degree of inhibition or neutralization of TF-binding capacity desired. Precise amounts of active ingredient required

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to be administered depend on the judgment of the
practitioner and are peculiar to each individual.
However, suitable dosage ranges are of the order of one
to several milligrams of active ingredient per

5   individual per day, preferably one to several
milligrams of active ingredient per kilogram bodyweight
of individual per day, more preferably about
0.05mg/kg/day to about 20mg/kg/day, and even more
preferably about 1mg/kg/day to about 5mg/kg/day, and

10   depend on the route of administration. Suitable
regimes for initial administration and booster shots
are also variable, but are typified by an initial
administration followed by repeated doses at one or
more hour intervals by a subsequent injection or other

15   administration. For example, a single bolus of about
500μg/kg may be administered (e.g., intravenously) for
therapeutic benefit.

Alternatively, continuous modes of administration,
such as via intravenous infusion, sufficient to

20   maintain concentrations of ten nanomolar to ten
micromolar in the blood are contemplated. In one
embodiment, a therapeutically effective amount for
intravenous administration is an amount sufficient to
produce plasma levels of the effective ingredient

25   (e.g., compounds which bind to TF, such as monoclonal
antibodies) of about 0.1μg/ml to about 10μg/ml are
contemplated. In another embodiment, administration of
an amount sufficient to produce plasma levels of said
molecules of about 1μg/ml to about 5μg/ml are

30   contemplated.

3.   Conjugates

Within the body, the primary mode of communication
and delivery of substances is via the circulatory
system. In general, the circulatory system comprises

35   the blood vascular system and the lymphatic system. Of

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particular interest are the capillaries.

Capillaries are typically comprised of simple
endothelial cells that connect the arterial and venous
sides of the circulatory system. Meshes of the
5   capillary network are present throughout the body,
varying in size and in shape in different tissues and
organs. The intensity of metabolism in a region
generally determines the closeness of the mesh.
Therefore, there is a close network in the lungs,
10   liver, kidneys, mucous membranes, glands, and skeletal
muscle, as well as in the grey matter of the brain.
The network has a large mesh and is sparse in tissues
such as tendons, nerves, smooth muscle, and serous
membranes.

15   The ability to transfer substances through the
wall of capillaries is referred to as permeability.
Permeability varies regionally and, under changed
conditions, locally.

In general, it is agreed that tumors must induce a
20   new blood supply if they are to grow beyond a diameter
of a few millimeters, and a great deal of attention has
been focused on the mechanisms by which tumors induce
angiogenesis. (For example, see Folkman, Adv. Cancer
Res. 43: 175-203 (1985).) Significant attention has
25   also been devoted to the anatomy and physiology of the
new blood vessels that come to supply tumors. (Id.)

It is generally agreed that tumor vessels are
anatomically heterogeneous structures. Often, they
consist of relatively undifferentiated channels, lined
30   by a simple endothelium and with fewer pericytes and
smooth muscle cells than would be expected of
comparably sized vessels in normal tissues. One
property of tumor vessels on which most investigators
agree is that, relative to normal vessels, tumor
35   vessels are hyperpermeable to circulating

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macromolecules. This observation demands explanation because of its obvious relevance to an understanding of the localization of monoclonal antibodies and tumoricidal drugs in solid tumors. (See, e.g., Dvorak,

5   et al., Am. J. Pathol. 133: 95-109 (1988).) Whereas small molecules pass freely through normal capillaries and other vessels with intact interendothelial cell junctions, the permeability of the normal vasculature to macromolecules is tightly regulated. Normally,

10   macromolecules are largely retained within the circulation and the small amounts that do escape are thought to do so by means of vesicular transport or by the formation of transient transcytoplasmic channels across endothelial cells. (See, e.g., Milici, et al.,

15   J. Cell Biol. 105: 2603-2612 (1987).) In inflammation, however, the escape of macromolecules is greatly increased; agonists such as histamine provoke a contraction of post-capillary endothelial cells, resulting in the formation of interendothelial cell

20   gaps through which macromolecules and even particulates may escape. This suggests, therefore, that tumor vasculature is "leaky".

Studies show that uptake of antibodies by tumor tissue correlates well with vascular permeability and

25   blood flow (Sands, et al., Cancer Res. 48: 188-193, (1988)). A similar study indicates that administration of a vasoactive agent may under some circumstances increase the perfusion of tumor relative to other tissues and increase tumor uptake and concentration of

30   pharmaceuticals. (Bomber, et al., J. Nucl. Med. 27: 243-245 (1986)).

Thus, it may also be appropriate to enhance delivery of the immunoglobulins of the present invention by administering them in conjunction with, or

35   by conjugating them to, other therapeutically useful

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agents. For example, the anti-huTF mAbs of the present
invention may be administered in conjunction with one
or more vasoactive agents.

Preferably, immunoglobulin-agent conjugates would
5   retain the ability to bind with antigen; more
preferably, they would also fail to bind with any
component of blood or normal, intact, non-inflamed
endothelium. It is also preferable that such
conjugates would show little or no tendency to pass
10   across the endothelium of normal blood vessels from
blood into tissues. It is also conceivable that anti-
huTF mAbs of the present invention may be conjugated to
other antibodies or fragments thereof, such as mAbs
that are specifically targeted to an antigen present on
15   a specific type of tumor. Methods for making and using
such conjugates are known in the art and will thus not
be discussed at length herein.

Vasoactive agents which improve the extravascular
penetration and binding of monoclonal antibodies (as
20   well as other drugs or molecules) may also be useful
and may be administered in conjunction with, or
conjugated to, the immunoglobulins of the present
invention. Although conjugates other than vasoactive
agent-immunoglobulin conjugates are also contemplated
25   by the present invention, immunoglobulin-vasoactive
agent conjugates (or "immunoconjugates") shall be
discussed herein as exemplary. It is expressly to be
understood that the present invention is not limited to
such conjugates.

30   Immunoconjugates useful as disclosed herein may be
prepared by various methods including genetic
approaches, or by covalently or otherwise linking a
selected clinically useful immunoglobulin to a selected
biologically active agent which is inflammation
35   provoking, and preferably vasoactive. The linking

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agent and the chemical procedure of assembling the immunoconjugate should be selected and carried out so as not to compromise the effectiveness of the antibody in binding to its target or the effectiveness of the

5   vasoactive agent in stimulating natural defense mechanisms.

Vasoactive agents as described herein are typically used to increase the flow of blood and/or the vessel permeability in the tumor so as to improve the

10   extravascular penetration and binding of monoclonal antibodies and other drugs or molecules _in vivo_. They may act directly by increasing the volume of tumor blood flow or the degree of tumor blood vessel "leakiness," or indirectly by inducing an inflammatory

15   immune response at the tumor site.

Inflammation can be induced by chemotactic factors which attract polymorphonuclear leukocytes, macrophages, eosinophils, basophils, mast cells, T-cells and other cells associated with inflammation.

20   These cells, when stimulated, secrete immunomodulatory factors which then act on the tumor blood flow and blood vessel permeability to increase the percent of the injected dose penetrating and binding to the tumor.

Vasoactive agents having the described reactivity

25   at the tumor site and suitable for linking to monoclonal antibodies in an immunoconjugate are found in several biochemical classes, including peptides, carbohydrates, and lipids, and their derivatives. Examples of useful agents include tachykinins,

30   leukotrienes, anaphylatoxins, lymphokines, inflammagens, human eosinophil acidic tetrapeptides, proteases, platelet-activating factor (PAF), prostaglandins, prostaglandin derivatives, histamine, and vasoactive carbohydrate compounds such as glucan.

35   In all instances, vasoactive agents are selected

according to availability and applicability to the
stated goals of therapy or study.

　　　The structural link between the immunoglobulin and
the agent to which it is conjugated, and the chemical
5　　method by which they are joined, should be chosen so
that the binding ability of the immunoglobulin and the
biological activity of the agent, when joined in the
conjugate, are minimally compromised. Some of the
available conjugation methods known in the art include
10　 the following.

　　　ECDI (1-ethyl-3-(3-dimethylaminopropyl)
carbodiimide) produces crosslinks between the antibody
and a second molecule (or conjugant), regardless of
either molecule's orientation. Conjugates are derived
15　 by condensation of the antibody and conjugant under
acidic conditions with ECDI. This method provides a
rapid and simple means of conjugation. (See
Goodfriend, et al., Science 144: 1344-1346 (1964).)

　　　N-succinimidyl 3-(2-pyridyldithio) propionate
20　 (SPDP) is a heterobifunctional reagent which introduces
thiol groups to the terminal amino of proteins, and has
been used in a number of immunoconjugates. (Carlsson,
et al., Biochem. J. 173: 723-737 (1978).)   The
cis-aconitic linkage described by Shen, et al has the
25　 characteristic of releasing a conjugant at low pH, such
as in a secondary lysosome following endocytosis of the
receptor-bound antibody molecule. The method allows
conjugation to the carbohydrate side groups of the
antibody molecules. (Shen, W.-C., and Ryser, Biochem.
30　 Biophys. Res. Comm. 102(3): 1048-1054 (1981).)

　　　Periodate oxidation can be used to oxidize and
cleave carbon-carbon bonds in a sugar ring. The
exposed terminal groups can then link to NH_2 groups on
proteins in a Schiff base linkage which is reduced with
35　 $NaBH_4$. (Kitao and Hattori, Nature 265, January 6, pp.

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81-82 (1977).) N-hydroxysuccinimide (NHS) activates a
terminal COOH group, for example, of a peptide, to form
an active ester derivative that can be covalently
coupled to the protein of the monoclonal antibody.

5   This method has been used to attach 30 molecules of
chlorambucil/antibody with little loss of binding
activity. (Smyth, et al., J. Natl. Cancer Inst. 76(3):
503-510 (1986).)

     As an alternative method to the chemical linkage
10   of biologically active agents (e.g., vasoactive agents)
to immunoglobulins of the present invention, the
genetic sequence of the agent can be engineered into
the genetic sequence of the immunoglobulin. For
example, the genetic sequence of a vasoactive peptide
15   may be engineered into that of one of the antibodies of
the present invention.

     The dose of vasoactive agent or immunoconjugate to
be given is based on criteria of medical judgment and
experience, both objective and subjective. However, an
20   adequate measure of an effective dose is that amount
required to increase localization of a subsequently- or
concurrently-administered diagnostic or therapeutic
agent (such as the immunoglobulins of the present
invention) to an extent which improves the clinical
25   efficacy of therapy, or accuracy of diagnosis, to a
statistically significant degree. Comparison is
typically made between treated and untreated tumor host
animals to whom equivalent doses of the diagnostic or
therapeutic agent are administered.

30   The immunodiagnostic dose may comprise an
immunoglobulin having a specificity for huTF and having
a label which is detectable in vivo. In a preferred
embodiment, this label comprises a radioactive isotope.
The immunotherapeutic dose may similarly comprise a
35   clinically useful immunoglobulin such as those

described herein, administered in conjunction with a
vasoactive agent, or alone. This immunoglobulin may
further be attached to a tumoricidal agent, for
example, a radioisotope, a chemotherapeutic drug or a
5   toxin.

     C.   Hybridomas

Methods for producing polyclonal anti-polypeptide
antibodies are well known in the art. See, e.g., U.S.
Patent No. 4,493,795 to Nestor, et al. A monoclonal
10   antibody, typically containing Fab and/or F(ab')$_2$
portions of useful antibody molecules, can be prepared
using the hybridoma technology described in Antibodies:
A Laboratory Manual, Harlow and Lane, eds., Cold Spring
Harbor Laboratory, New York (1988), which is
15   incorporated herein by reference. Briefly, to form the
hybridoma from which the monoclonal antibody
composition is produced, a myeloma or other self-
perpetuating cell line is fused with lymphocytes
obtained from the spleen of a mammal hyperimmunized
20   with huTF or a receptor-binding portion thereof.

Methods for producing hybridomas capable of
producing (secreting) antibody molecules having a
desired immunospecificity -- i.e., having the ability
to immunoreact with a particular protein and/or
25   polypeptide -- are well known in the art. Particularly
applicable is the hybridoma technology described by
Niman, et al., PNAS USA 80: 4949-4953 (1983).
Preferred hybridomas are those which secrete antibodies
capable of immunoreacting with huTF. Even more
30   preferred are those which secrete antibodies capable of
immunoreacting with an huTF epitope, and more
particularly, with the epitope located at or near the
carboxy terminus of native huTF and defined or
encompassed by amino acid residue nos. 106-219.
35   The hybridomas of the present invention are

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characterized as producing antibody molecules that
immunoreact with huTF. Such antibodies may further be
capable of immunoreacting with polypeptide analogs TF
epitopes, e.g. p204-226. Preferred methods of

5   producing and screening antibodies useful according to
the present invention are set forth in U.S. Patent No.
5,110,730 to Edgington, et al. and is incorporated
herein by reference. (Also see U.S. Patent No.
5,223,427 to Edgington, et al., which is also

10   incorporated herein by reference.)

        Exemplary hybridomas (and antibodies produced
thereby) include those described in U.S. Pat. App. No.
5,110,730. Especially preferred hybridomas are those
identified by ATCC accession numbers HB9382 and HB9381

15   (ATCC, Bethesda, MD). Other preferred hybridomas are
those identified in Table 1 of Morrissey, et al.,
Thromb. Res. 52: 247-261 (1988). The monoclonal
antibodies listed in Table 1 of that reference were
prepared essentially as follows.

20   The immunogen, human brain TF isolated by the
factor VII-affinity method, was lyophilized and
extracted twice with 80% acetone to remove detergent.
After drying under a stream of nitrogen, TF was
dissolved in normal saline and emulsified with adjuvant

25   as required. Denatured TF was prepared by boiling for
5 minutes in TBS containing 0.5% SDS and 1% 2-
mercaptoethanol. Carrier mouse serum albumin was added
followed by acetone extraction (as described in
Morrissey, et al., supra).

30   Female BALB/c mice (from The Scripps Research
Institute breeding colony) were injected subcutaneously
with 5μg of huTF in Ribi adjuvant (monophosphoryl lipid
A, trehalose dimycolate, squalene and Tween-80
emulsion; from Ribi Immunochem Research, Inc.,

35   Hamilton, MT). Mouse TF8 received native TF and mouse

TF9 received denatured TF. Fourteen days later, each
mouse was again injected subcutaneously with 5μg of
denatured huTF in Ribi adjuvant, and again on day 28
intraperitoneally with 33μg of native huTF in complete
5   Freund's adjuvant (Difco, Detroit, MI). On days 56 and
57, mouse TF8 received 15μg of native TF in saline
intravenously, followed on day 58 by harvest of the
spleen for hybridoma production. On days 154 and 155,
mouse TF9 received 10μg of native TF intravenously
10   prior to spleen harvest on day 156. Fusion of spleen
cells to the P3Ag8.653.1 myeloma cell line and
isolation of hybridomas were performed as described in
Soule, et al., PNAS USA 80: 1332-1336 (1983) and Kohler
and Milstein, Nature 256: 495-497 (1975). Hybridomas
15   from fusion TF8 were screened against native TF on dot-
blots and also against native ^{125}I-TF in the
radioimmunoassay. The hybridomas from fusion TF9 were
screened against reduced, denatured TF on western blots
and also against native ^{125}I-TF in the radioimmunoassay.
20   Each hybridoma was recloned at least twice.
Isotypes were determined using an ELISA test (Hyclone
Laboratories, Logan, UT). Hybridoma culture
supernatants were used for most experiments with these
antibodies. When required, IgG was purified from
25   ascites fluid using affinity chromatography on
immobilized S. aureus protein A (MAPS II System; BioRad
Laboratories, Richmond, CA).
     Hybridomas TF8-5G9 and TF9-6B4 were deposited
pursuant to Budapest Treaty requirements with the ATCC
30   on March 27, 1987, and were assigned accession numbers
HB9382 and HB9381, respectively. (See also Ruf, et
al., Thromb. Haemost. 66: 529-533 (1991) and Morrissey,
et al., Thromb. Res. 52: 247-261 (1988).)
     Monoclonal antibody TF9-10H10, which does not
35   exhibit the presently-preferred immunospecificity, is

useful as disclosed herein. Antibody TF9-10H10 is
secreted by hybridoma TF9-10H10, which was deposited
with the ATCC on March 27, 1987, pursuant to Budapest
Treaty requirements, and was assigned accession number
5   HB9383.

D.   Xenograft Model

Metastasis is a multi-step process that has
specific requirements which must be met by both tumor
cells and host factors, in order for the process to be
10   completed successfully. Some studies have attempted to
correlate the proteases of the coagulation cascade with
hematogenous metastasis, but until the present
disclosure, it was not clear how and where this
proteolytic activity is generated in the metastatic
15   process.

The study of a xenograft model for hematogenous
melanoma metastasis allows us to distinguish the
contribution of tumor cells to the activation of
coagulation from that of host cells, because the
20   antibodies that were used to block procoagulation
activity are highly specific for human TF (Ruf, et al.,
Thromb. Haemost. 66: 529-533 (1991)). Evaluating a
small panel of human cell lines, it has now been
discovered that the expression of TF increases in
25   melanoma progression and, in particular, that cell
lines derived from melanoma metastases have potent
procoagulant activity that is entirely TF-dependent.
Procoagulant activity not only seems to be required for
M24met metastasis but for hematogenous metastasis of
30   other melanoma cell lines. The presently-described
experiments using the xenograft model demonstrate that
TF function is required for melanoma cells to initiate
or undergo hematogenous metastasis.

It is now reported herein that inhibition of TF
35   activity on the surface of metastatic melanoma cell

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lines (e.g., M24met and C8161) with specific monoclonal
antibodies inhibits experimental pulmonary metastasis
in xenograft models. The number of tumor foci in the
lungs is significantly reduced ($p<0.002$) after exposing
5   tumor cells to the monoclonal antibody TF8-5G9 or its
Fab fragment, which bind to human TF and inhibit TF
function. There was, however, no effect on metastasis
when cells were exposed to the monoclonal antibody TF9-
10H10, which is directed to a partly-overlapping
10   epitope on TF, but which does not inhibit TF function.
Monoclonal antibody TF9-10H10 is further described and
characterized in U.S. Patent No. 5,110,730 to
Edgington, et al. (which has been incorporated herein
by reference).

15   Distribution and fate of radiolabeled tumor cells
after I.V. injection indicated at least two phases of
melanoma cell arrest in the microvasculature of the
lungs. Thus, initial adherence is not influenced by
specific inhibitors of tumor cell TF and is apparently
20   not sufficient for prolonged tumor cell survival.
Inhibition of TF -- and thus, inhibition of the local
generation of proteolytic activity -- abolishes
adherence of melanoma cells at least up to four hours
after injection. The within-disclosed data also
25   suggest that early and local generation of proteases --
e.g., factor IXa, factor Xa, thrombin -- is required
for prolonged adhesive interaction and/or trans-
migration of melanoma cells across the endothelium
leading to the growth of metastatic foci.

30   E.   <u>Cell Lines and Tissue Culture</u>
Normal human epidermal melanocytes (NHEM) were
purchased from Clonetics (San Diego, CA). They were
maintained in melanocyte growth medium (MGM -
Clonetics, San Diego, CA) and used prior to the tenth
35   passage. The human melanoma cell line WM35 has been

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established from an early primary melanoma (Kath, et
al, <u>Cancer Therapy Control 1</u>: 179-191 (1990)). WM35
was maintained in MCDB153/L15 medium supplemented with
insulin (5 μg/ml) and two percent fetal bovine serum
5 (FBS). The human melanoma cell line M24met has been
described in detail (Mueller, et al, <u>Cancer Res. 51</u>:
2193-2198 (1991)). The M24met line was derived from
M24, which was established from a lymph node metastasis
(Guiliano, et al, <u>Cancer Immunol. Immunother. 10</u>: 243-
10 249 (1981); Tsuchida, et al., <u>J. Natl. Cancer Inst. 78</u>:
55-59 (1987)). The human melanoma cell line C8161 was
also derived from a metastatic melanoma (Welch, et al.,
<u>Int. J. Cancer 47</u>: 227-237 (1991)). M24met and C8161
were maintained in RPMI 1640 tissue culture medium
15 supplemented with 10% FBS (Sigma, St. Louis, MO). WM35
and M24met were cultured on tissue culture plastic that
was pretreated with 1% gelatin to enhance cellular
attachment. All cells were routinely tested from
mycoplasma contamination.

20   F.   <u>Monoclonal Antibodies</u>

        The murine anti-human TF monoclonal antibodies
TF8-5G9 and TF9-10H10 have been previously
characterized (Ruf, et al., <u>Thromb. Haemost. 66</u>: 529-
533 (1991); Ruf, et al., <u>Biochem. J. 278</u>: 729-733
25 (1991)). Both mAbs are of IgG1 isotype. The IgG1 mAb
to the SV40 large T viral antigen (TIB115, ATCC,
Rockville, MD) was used as isotype matched irrelevant
control. The mAbs were purified on protein A, and Fab
fragments of mAb TF8-5G9 were produced by papain
30 digestion (Ruf, et al., <u>Thromb. Haemost. 66</u>: 529-533
(1991)), followed by ion exchange chromatography to
remove uncleaved IgG and F$_c$ fragments.

        G.   <u>Functional Analysis of TF</u>

        TF functional activity was analyzed in a plasma
35 coagulation assay after octyl-glucopyranoside lysis of

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cell pellets, as previously described (Ruf, et al., _J._
Biol. Chem. _267_: 6375-6381 (1992)). Briefly, cells
were lysed at 2 X 10^6 cells/ml with 15mM octyl-β-D-
glucopyranoside in HEPES-buffered saline (HBS) at 37°C

5   for 20 minutes, followed by a 3-fold dilution with HBS
(see also Drake, et al., _J. Cell. Biol. 109_: 389-395
(1989)). Clotting times were determined for the cell
lysates in a one-stage clotting assay (equal volumes of
sample, plasma, and 20mM $CaCl_2$) and converted to units

10   based on a calibration curve using purified and
phospholipid-reconstituted TF. The clotting times
obtained in the one-stage clotting assay were converted
into units of TF activity using a calibration curve
based on serial dilutions of a cell line expressing

15   wild-type TF, as previously described in detail
(Rehemtulla, et al., _J. Biol Chem. 266_: 10294-10299
(1991)).

     TF was quantified by converting clotting times
into procoagulant activity units based on a calibration

20   curve using purified and phospholipid reconstituted
recombinant TF. The activity which yields a 50s
clotting time was defined as the unit 1U/ml. The
procoagulant activity was demonstrated to be TF-
specific by blocking TF function in the cell lysates by

25   a 30-minute preincubation ·with mAb TF8-5G9 (30μg/ml).
The functional activity of human recombinant TF was
compared in mouse plasma (Sigma, St. Louis, MO) and
pooled normal human plasma using a standard coagulation
assay.

30   H.   Analysis of Cell Surface TF
     The cell surface expression of TF was determined
via flow cytometry, as described herein. Melanoma
cells (10^6 cells/tube) were suspended in phosphate
buffered saline (PBS) with 50 μg/ml mAb for 45 minutes

35   on ice. Cells were washed, and bound primary mAb was

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detected by incubation with goat anti-mouse IgG
antibody labeled with fluorescein for 45 minutes on
ice. After washing to remove unbound secondary
antibody, cells were analyzed with a FACS 440 flow
5   cytometer. Cell surface TF was quantified on M24met
melanoma cells by radioligand analysis. The mAb TF8-5G9
was labeled to a specific activity of 5 nCi (185 Bq)/ng
with Na^{125}I (Amersham, Arlington Heights, IL) with
Enzymobeads (BioRad, Richmond, CA). Radioligand
10 binding analysis with the radiolabeled mAb was
performed on melanoma cell monolayers as described
(Mueller, et al., _J. Immunol. 144_: 1382-1386 (1990)).
The number of TF molecules per cell and the
dissociation constant of the ligand were determined by
15 Scatchard plot analysis.

I.   TF Expression

Another aspect of the present invention discloses
methods of identifying compounds which may be useful as
inhibitors of cell-surface expression of TF. In
20 general, an assay for identifying such compounds may
comprise the steps of: (a) culturing tumor cells; (b)
admixing said tumor cells with a test compound,
preferably one that is not cytotoxic; (c) incubating
the resulting admixture for a predetermined period of
25 time; and (d) determining the amount of TF expressed
(e.g., per cell).

Cell surface expression may be impacted at several
levels; depending upon the manner in which one intends
to disrupt cell surface expression, a test compound
30 will have different characteristics. For example, if
one intends to alter the rate of transcription or
translation of a TF gene and/or sequences involved in a
TF-regulatory pathway, a "test" compound may comprise a
nucleotide sequence such as an antisense nucleotide
35 sequence. Alternatively, it may comprise a molecule

which binds to at least a portion of the TF-encoding
sequence or a nucleotide sequence which affects the
rate or frequency of transcription of a TF-encoding
sequence, such as the TF promoter or regulatory

5   enhancer elements. Such molecules would thus include
transcription repressors. Other potentially-useful
molecules include molecules that mimic growth factors
or otherwise compete with same for binding sites on
corresponding cognate cellular receptors, to inhibit

10   the initiation of transcription of the TF gene. (See,
e.g., Edgington, et al., Thromb. Haem. 66: 67-79
(1991); Lau, et al., PNAS USA 84: 1182-6 (1987).)
Molecules which alter the post-translational regulation
of TF expression are also potentially useful as

15   therapeutic agents.

Should one desire to modulate TF cellular
processing mechanisms, potentially-useful molecules
would likely include those that impact sequestration,
for example. Other potentially-useful candidates

20   include molecules which alter TF degradation
mechanisms.

Additional molecules or compounds potentially
useful as modifiers of expression levels of TF include
such substances as interleukins (e.g., IL-1) and other

25   cytokines, as well as growth factors and substances
such as tumor necrosis factor (TNF), which exhibit
cytokine-like effects. For example, in Bevilaqua, et
al., J. Exp. Med. 160: 618-623 (1984), the induction of
biosynthesis and cell surface expression of

30   procoagulants via use of IL-1 was described. (Also see
Edgington, et al., Thromb. Haem. 66: 67-79 (1991).)
Other selected agonists such as endotoxins (e.g. LPS),
antigen-antibody complexes, and vascular permeability
factor (VPF) may induce expression of TF and may thus

35   be useful as disclosed herein. (See Morrissey, et al.,

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in <u>Oxford Surveys on Eukaryotic Genes</u>, MacLean (ed.), Oxford Univ. Press, 1989, p. 67-84; Levy, et al., <u>J. Exp. Med. 154</u>: 1150-1163 (1981); Keck, et al., <u>Science 246</u>: 1309-1312 (1989).)

5   EXAMPLES

The following examples are intended to illustrate, but not limit, the present invention.

<u>Example I</u>

<u>Experimental Metastasis Assay</u>

10   Human melanoma cells (0.5 to 1.0 X 10^6 cells in 200μl PBS) were injected into the lateral tail vein of 6-8 week old female C.B-17 <u>scid/scid</u> mice (from The Scripps Research Institute Rodent Breeding Facility, La Jolla, CA). Prior to injection, cells were incubated

15   for 45 minutes at room temperature. Buffer controls were incubated in PBS and a total of 1mg mAb or Fab fragment was present in the experimental groups. When a 2mg total dose was administered, mice received a second intravenous injection of 1mg mAb after 24 hours.

20   Twenty-one days later, these animals were sacrificed and necropsy was performed. All major organs were systematically examined for the presence of tumors. The lungs were removed and their weight determined prior to fixation in Bouin's solution. Tumor foci on

25   the lung surface were counted under a low magnification microscope. Data were analyzed for statistical significance by the non-parametric Wilcoxon rank sum test.

<u>Example II</u>

30   <u>Determination of Distribution of Radiolabeled</u>

<u>Melanoma Cells After Intravenous Injection</u>

The initial localization of radiolabeled tumor cells to organs was analyzed using the methodology described by Esumi, et al. (<u>Cancer Res. 51</u>: 4549-4556

35   (1991)). Cells were cultured for 24 hours in the

presence of 0.1 μCi (3.7 kBq)/ml 5-(^{123}I)-iodo-2'-
deoxyuridine (Amersham, Arlington Heights, IL)
resulting in the incorporation of 50,000 cpm per 10^6
M24met cells. This had no effect on the viability of

5   the cells. After incubation with mAb or saline for 45
minutes, 10^6 cells in 200μl were injected into the
lateral tail vein of female scid mice. Mice were bled
from the orbital sinuses (50μl/mouse) and sacrificed at
10 min., 90 min., 240 min. and 24 hours post-injection.

10   Lungs, livers, spleens, and kidneys were collected and
washed in 70% ethanol for 3 days prior to gamma-
counting.

Example III
Assay for Expression of TF in Metastatic Melanomas

15   The expression of TF on cultured melanocytes and
melanoma cell lines was analyzed with a sensitive
coagulation assay (see Fig. 1). The various cell lines
used are shown on the vertical axis; procoagulant
activity expressed in mU/10^6 cells is shown on the

20   horizontal axis. TF functional activity was analyzed
in a plasma coagulation assay after octyl-
glucopyranoside lysis of cell pellets of normal human
epidermal melanocytes (NHEM) and melanoma cell lines
WM35, M24met, and C8161. TF specific activity was

25   assessed after blocking TF in cell lysate by a 30
minute preincubation with mAb TF8-5G9 (30μg/ml). Means
from four independent experiments are illustrated in
Figure 1.

The procoagulant activity of the cell lysates was

30   shown to be TF-specific by inhibition with anti-TF
monoclonal antibodies (see Fig. 1). Both cultured
human melanocytes and the cell line WM35, which was
derived from a primary non-metastatic melanoma (Kath,
et al., Cancer Therapy Control 1: 179-191 (1990)),

35   expressed TF at very low levels. In contrast, the

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melanoma lines M24met (Mueller, et al., <u>Cancer Res. 51</u>:
2193-2198 (1991)) and C8161 (Welch, et al., <u>Int. J.</u>
<u>Cancer 47</u>: 227-237 (1991)), which were established from
melanoma metastases, had a more than 1000-fold higher

5   level of TF expression. When cell lysates of M24met
and C8161 were electrophoretically separated and
analyzed by Western blotting with mono-specific
antibodies to TF, a single 47 kDa protein was
identified under reducing conditions, consistent with

10   fully glycosylated and properly processed human TF
(Morrissey, et al., <u>Thromb. Res. 50</u>: 481-493 (1988);
data not shown). High level expression of TF thus
characterizes these metastatic melanoma cells.

Indirect immunofluorescence was used to assess

15   whether TF is expressed on the tumor cell surface or is
sequestered in intracellular compartments where it
would likely be of minor functional significance <u>in</u>
<u>vivo</u>. Specific immunoreactivity of mAbs TF8-5G9 and
TF9-10H10 is demonstrated by flow cytometry using non-

20   permeabilized M24met or C8161 cells (see Fig. 2).

Figures 2A and B illustrate the surface expression
of TF on metastatic melanoma cell lines. In each
instance, the number of cells is plotted on the
vertical axis, while fluorescence intensity is shown on

25   the horizontal axes. Indirect immunofluorescence and
flow cytometry were used to demonstrate TF antigen on
the surface of M24met (Fig. 2A) and C8161 (Fig. 2B)
melanoma cell lines. Cells were incubated with 50μg/ml
isotype matched control Ab (TIB115; solid line), or

30   anti-TF mAb TF8-5G9 (bold line) and TF9-10H10 (dotted
line), respectively. Bound mAb was visualized by a
secondary goat anti-mouse IgG conjugated to fluorescein
isothiocyanate. Results of representative experiments
are shown in each of 2A and 2B.

35   Additional anti-TF mAbs to non-overlapping

epitopes provided similar results (data not shown).
The cell surface expression of TF on M24met cells was
approximately 3.5- to 4-fold lower than on C8161 cells,
consistent with the relative functional TF activities
5   of these cell lines determined in the coagulation
assay. Cell surface expression on M24met cells was
also quantified by radioligand binding analysis with
radiolabeled mAb TF8-5G9. Scatchard analysis
demonstrated saturable binding to 64,000 ± 28,000 sites
10   per cell with a dissociation constant of 197 ± 5 pM
(mean ± standard deviation, n=3). These data are
consistent with predominant cell surface expression of
TF on the cells. TF therefore represents the major
initiator of the coagulation pathways on the surface of
15   these metastatic melanoma cells.

<div align="center">Example IV</div>

<div align="center">Specific Inhibition of Human TF in Murine Blood</div>

To explore whether the presence of functional TF
on the melanoma cell surface contributes to metastatic
20   tumor dissemination, a xenograft model in which human
melanoma cells are injected intravenously into mice
with severe combined immune deficiency (scid) was
chosen. Species compatibility was assessed with
recombinant human TF reconstituted into phospholipid
25   vesicles.

Figure 3A illustrates functional activity of human
TF in human and mouse plasma. Clotting time in seconds
is plotted on the vertical axis, whereas the amount of
human TF (in ng/ml) is shown on the horizontal axis.
30   The ability of phospholipid reconstituted recombinant
human TF to initiate coagulation in mouse plasma (open
circles) was evaluated in a standard clotting assay in
comparison to pooled normal human plasma (closed
circles).

35   Figure 3B shows that, in either case, a

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preincubation with 30μg/ml TF8-5G9 inhibited TF-induced
coagulation, whereas preincubation with TF9-10H10 did
not. In Fig. 3B, procoagulant activity (mU/ml) is
shown on the vertical axis. On the horizontal axis,
5   results of preincubation of human vs. mouse plasma with
30μg/ml control (TIB115), 10H10 (TF9-10H10) or 5G9
(TF8-5G9) mAb is shown.

Human TF triggered coagulation with the same
efficiency in both human and mouse plasma (Fig. 3),
10   demonstrating that the xenograft model adequately
reflects the interaction of tumor cell TF with the
host's blood. Based on analysis in a purely human
system, the monoclonal antibodies TF8-5G9 and TF9-10H10
have distinct functional characteristics, although they
15   are directed to partially overlapping epitopes (Ruf, et
al., Biochem. J. 278: 729-733 (1991)). Whereas mAb
TF9-10H10 does not inhibit binding of factor VIIa and
function of the TF·VIIa complex, mAb TF8-5G9 is a
potent and rapid inhibitor of human TF in human plasma
20   due to its ability to compete with substrate access to
the TF·VIIa complex. In addition, this mAb does not
inhibit TF from various non-human primates (except
chimpanzee) or any lower mammal species tested to date
(Morrissey, et al., Thromb. Res. 52: 247-261 (1988)),
25   thus providing a highly-specific tool to selectively
block human TF function in the xenograft model. Both
the inhibitory mAb TF8-5G9 and the non-inhibitory mAb
TF9-10H10 had identical functional characteristics
whether in human or mouse plasma (Fig. 3). The use of
30   the human TF-specific mAb TF8-5G9 thus allows selective
inhibition of melanoma cell TF function without
interfering with murine TF expressed on host cells.

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Example V
Melanoma Cell TF Function is Required
for Hematogenous Metastasis

Intravenous injection of M24met metastatic

5   melanoma cells into scid mice reliably produces a large
number of metastases in the lungs. Table 2 illustrates
the effect of functional inhibition of TF, in an
experimental pulmonary metastasis model. In that
table, the number of metastases per mouse in each

10   experiment is plotted against the total dose of
monoclonal antibody (or buffer solution only)
administered to each subject.

As illustrated in Table 2, M24met or C8161 cell
suspensions were incubated in buffer with or without

15   1mg of mAb TF8-5G9 mAb or its Fab fragment, mAb TF9-
10H10, or isotype control mAb TIB115 (identified as
IgG_1 in Table 2), for 45 minutes prior to injection
into the lateral tail vein of scid mice. When a 2mg
dose was administered, mice received a second

20   intravenous injection of 1mg mAb after 24 hours. After
21 days, all animals were sacrificed, their lungs
fixed, and the number of visible tumor nodules on the
lungs was counted. The probability of no difference
(p) between buffer control and mAb-treated groups is

25   indicated based on the non-parametric Wilcoxon rank sum
test.

Table 2

EFFECT OF FUNCTIONAL INHIBITION OF TF ON
EXPERIMENTAL PULMONARY METASTASIS

Experiment 1: 1×10^6 M24met

mAb (total dose)	No. Metastases/Mouse (total in both lungs)						p
none	314	353	356	375	384	402	-
TF8-5G9 (1mg)	14	16	27	42	94	153	<0.0022
TF9-10H10 (1mg)	279	293	346	363	375	377	0.309
IgG$_1$ (1mg)	281	291	292	327	368	424	0.24

Experiment 2: 1×10^6 M24met

mAb (total dose)	No. Metastases/Mouse (total in both lungs)						p
none	41	86	145	175	196	221	-
TF8-5G9 (1mg)	0	0	0	0	0	1	<0.0022
TF8-5G9 (2mg)	0	0	0	0	0	0	<0.0022
TF8-5G9 Fab (2mg)	0	2	5	6	15	16	<0.0022

Experiment 3: 5×10^6 C8161

mAb (total dose)	No. Metastases/Mouse (total in both lungs)						p
none	20	21	37	65	88	170	-
TF8-5G9 (2mg)	0	0	0	2	3	4	<0.0022
TF9-10H10 (2mg)	2	3	15	21	56	63	0.09

The number of experimental pulmonary metastases
was significantly reduced when mice were infused with
M24met cells in the presence of 1mg TF8-5G9, in
comparison to a control group injected with M24met
5   cells in buffer (see Table 2). Mice receiving TF8-5G9
also had a significant reduction in metastases when
compared to animals treated with an irrelevant isotype
matched control mAb (TIB115) or the anti-TF mAb TF9-
10H10, which binds TF with high affinity without
10 blocking its function. In an independent experiment,
the anti-metastatic effect of TF8-5G9 was reproduced.
Furthermore, Fab fragments of TF8-5G9 effectively
inhibited M24met metastasis. This finding further
excludes F_c-mediated effects, though considered
15 unlikely because of the lack of anti-metastatic effects
of the anti-TF mAb TF9-10H10 which is of the same
isotype as TF8-5G9. The inhibition by Fab fragments
was slightly less effective compared to the intact mAb;
this may be a result of an observed 2- to 3-fold poorer
20 affinity of Fab fragments of TF8-5G9 (Ruf, et al.,
Thromb. Haemost. 66: 529-533 (1991)). Blocking of TF
function by mAb or Fab fragments, but not binding of a
non-inhibitory mAb to the same receptor, reduces
pulmonary metastasis consistent with a requirement for
25 TF function in hematogenous metastasis of tumor cells.
        The role of TF in metastasis was further evaluated
with another metastatic melanoma cell line which
exhibits high levels of TF expression. Compared to
buffer control, mAb TF8-5G9 significantly inhibited
30 experimental lung metastasis of C8161 melanoma cells.
(See Table 2, Experiment 3.) Similar to M24met
melanoma cells, the non-inhibitory mAb TF9-10H10 did
not significantly reduce melanoma metastasis. However,
there was an apparent decrease in the number of
35 pulmonary metastases when compared to the buffer

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control group. This difference, which is not
statistically significant, may indicate a slight effect
of the 3.5- to 4-fold higher number of TF sites and
consequently bound mAb molecules on the surface of

5   C8161 compared to M24met cells which might elicit some
F_c-mediated host defense mechanisms. These data
demonstrate that only functional inhibition of TF
results in a significant reduction of pulmonary
metastasis, providing evidence that two independently

10   established metastatic melanoma cell lines require TF
function for efficient hematogenous metastasis.

Example VI
TF Function is Required for
Prolonged Adherence of Melanoma Cells

15   The distribution of intravenously injected
radiolabeled tumor cells was studied in order to assess
whether initial arrest in various organs and
persistence of tumor cells is influenced by blocking of
TF function (Fig. 4). Figures 4A and 4B illustrate the

20   distribution of radiolabeled M24met cells after
intravenous injection into scid mice. On the vertical
axis, time in minutes (4A) or hours (4B) is shown. On
the horizontal axis, the percent of injected dose is
illustrated, in liver, blood, and lungs. In 4A, p =

25   0.018; in 4B, p = 0.005.
           Cells were labeled with 5-(^{125}I)-Iodo-2'-
deoxyuridine for 24 hours, washed and incubated for 45
minutes with 1mg monoclonal antibody TF8-5G9 ("open" or
white bars) or buffer control ("closed" or colored

30   bars). Radiolabeled M24met cells were injected into
the tail vein of scid mice. Mice were bled and
sacrificed at different time points, as shown. Means
and standard deviations are based on four animals per
group at each time interval. The probability of no

35   difference occurring between groups at a given time was

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assessed with the Wilcoxon rank sum test.

Inhibition of TF function on M24met cells had no
effect on the rapid clearance of tumor cells from the
blood, when compared to the buffer control. As early
5   as 10 minutes after injection, only 1% of infused tumor
cells remained per ml of blood, and radiolabeled cells
were virtually absent after 24 hours in all animals.
In the livers of mice treated with the anti-TF mAb TF8-
5G9, more tumor cells were initially arrested in
10 comparison to the controls. This may reflect increased
clearance of a small fraction of the antibody coated
cells by the reticuloendothelial system of the liver.
Only traces of radiolabel (< 0.25% of the total dose)
were detected in spleens and kidneys at all time
15 points, with no differences in mAb-treated or untreated
animals.

The majority of tumor cells rapidly localized to
the lungs with no difference between the anti-TF mAb-
treated and the untreated control group. The
20 radiolabel in the lungs dropped from 75-80% of total
tumor cells at 10 minutes after injection to 55-60% of
the tumor cells 90 minutes after injection. This
indicates that the initial distribution and the
selective arrest in the lung vasculature is not
25 influenced by inhibition of TF function. However,
early attachment was not sufficient to dictate
successful implantation. Thus, inhibition of tumor
cell TF significantly reduced the number of cells
retained in the lungs four hours and 24 hours following
30 tumor cell infusion. At 24 hours, the radiolabel
equivalent to 170 tumor cells was present in the lungs
of animals receiving inhibitory anti-TF mAb compared to
15,000 cells in controls. Functional TF on the
melanoma cells thus appears to be required for
35 prolonged adherence of tumor cells at the site of

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initial arrest.

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Example VII

Discussion of the Results

of Examples I-VI
</div>

5   The malignant phenotype of cells typically
incorporates aberrations in growth control, as well as
their capacity to invade and metastasize. Critical
events in the metastatic process include (1) the
initial arrest and (2) stable implantation of tumor
10   cells in the microvasculature of target organs.

Various studies on the arrest of circulating tumor
cells discriminate between an initial phase of direct
contact between tumor cells and the vascular
endothelium and a second phase characterized by fibrin
15   generation and formation of a platelet thrombus in
association with the arrested tumor cells (Crissman, et
al., Cancer Res. 48: 4065-4072 (1988)). This indicates
that proteolytic activity is rapidly generated at the
site of tumor cell arrest, marking a second phase of
20   stabilized implantation at the endothelium.

It is demonstrated herein that generation of
proteolytic activity on the surface of metastasizing
human melanoma cell lines is triggered by TF, the major
initiating molecule of the coagulation cascades.
25   Indeed, the cellular expression of this molecule was
observed to parallel the progression of melanoma cells
to a metastatic phenotype. Whereas normal cultured
human melanocytes or a primary melanoma derived cell
line had only trace levels of functional TF, at least a
30   1000-fold higher cell surface expression was detected
on metastasizing human melanoma cells. Since all of
the cell lines were proliferating, expression of TF did
not reflect cell cycle difference, but appeared,
rather, to be the correlate of the metastatic
35   potential.

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The functional role of TF expressed on melanoma
cells was explored in an experimental metastasis model
in scid mice.   The mAb TF8-5G9, which selectively binds
and functionally neutralizes human TF in the xenograft

5   model, markedly reduced the number of pulmonary
metastases of two metastatic melanoma cell lines.   This
was shown to result from functional inhibition of tumor
cell TF rather that non-specific host defense effects
based on: (1) lack of reactivity of the murine anti-

10   human TF mAbs with host TF; (2) inhibition of
metastasis by Fab fragments of mAb TF8-5G9; and (3)
unaltered implantation of pulmonary metastasis when a
non-inhibitory mAb was bound to the same cell surface
receptor.   Inhibition of TF function did not interfere

15   with the initial arrest of the tumor cells in the lungs
of animals, but rather, abolished the prolonged
localization to this organ.   This study thus
establishes a requisite second phase in tumor cell
implantation which is dependent on tumor cell TF-driven

20   generation of coagulation serine proteases.

The initial arrest of tumor cells appeared not to
be dependent on the generation of proteolytic activity.
Direct interaction between tumor cells and endothelial
cells through constitutively expressed and lung-

25   specific endothelial cell adhesion molecules, such as
Lu-ECAM-1 (Zhu, et al., PNAS USA 88: 9568-9572 (1991))
may be responsible for this early attachment.   This
initial arrest, though necessary for the metastatic
process (Zhu, et al., J. Clin. Invest. 89: 1718-1724

30   (1992)), is not sufficient to establish metastases.   In
a second phase, TF on the tumor cell surface is
required to activate the coagulation cascades which,
among other serine proteases, ultimately generate
thrombin.   Inhibition of this protease in the early

35   phase of tumor seeding has indeed been shown to reduce

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pulmonary metastasis (Esumi, et al. (Cancer Res. 51:
4549-4556 (1991)). However, the metastatic process may
be dependent on various biological effects of thrombin
which are mediated through proteolytic activation of
5   the thrombin receptor (Coughlin, et al., J. Clin.
Invest. 89: 351-355 (1992)).

        Thrombin may trigger critical events in the early
phase of tumor cell implantation. Stimulation of
endothelial cells by thrombin results in rapid
10   expression of P-selectin (Pober, et al.,
Transplantation 50: 537-544 (1990)) for which ligands
are found on a variety of tumor cells (Aruffo, et al.,
PNAS USA 89: 2292-2296 (1992)). More delayed
expression of E-selectin upon stimulation of
15   endothelial cells may contribute to additional
cooperative adhesive interactions through carbohydrate
ligands associated with the melanoma cells (Burrows, et
al., Cancer Res. 51: 4768-4775 (1991)). The avidity of
adhesive receptors is modulated by signaling molecules
20   such as platelet activating factor (PAF) which is
synthesized by endothelial cells upon thrombin
stimulation (Zimmerman, Immunology Today 13: 93-100
(1992)). Melanoma-endothelial cell interaction in
metastasis may recapitulate the principles of
25   leukocyte-endothelial cell interactions in inflammation
(Butcher, Cell 67: 1033-1036 (1991)). This process is
characterized by an initial, transient and reversible
adhesion mediated by selectins, followed by more stable
adherence resulting from the up-regulation and
30   functional participation of leukocyte integrins and
their counter-receptors on the stimulated endothelial
cell.

        Additional events may be triggered by the initial
generation of proteolytic activity at the site of tumor
35   cell arrest. Fibrin generation may protect arrested

cells from host defensive mechanisms (Gunji, et al., Cancer Res. 48: 5216-5221 (1988)). Fibrin which is deposited locally serves as a matrix for the interaction of tumor cells through their adhesive receptors which may modify or induce the expression of genes relevant for invasion and tumor growth (Hynes, Cell 69: 11-25 (1992)). Thrombin may support invasion of tumor cells by degrading the subendothelial matrix either directly (Liotta, et al., Cancer Res. 41: 4629-4636 (1981)) or indirectly by inactivation of inhibitors for other tumor cell-associated proteases (Ehrlich, et al., J. Cell. Biol. 115: 1773-1781 (1991)). Proteolytic activation of the thrombin receptor (Coughlin, et al., J. Clin. Invest. 89: 351-355 (1992)) on tumor cells or host cells may modify their cellular responses and stimulate proliferation. Platelets are deposited upon thrombin stimulation at the sites of tumor cell arrest and the inhibition of platelet function results in inhibition of metastasis in sever al experimental models (Karpatkin, et al., J. Clin. Invest. 81: 1012-1019 (1988); Okoshi, et al., Cancer Res. 51: 6019-6024 (1991)). However, it is not known whether the stabilization of the initial fibrin clot (Weiss, et al., FASEB J. 2: 12-21 (1988)) or the release of pleiotropic growth modulators such as platelet-derived growth actor (PDGF), platelet factor 4 (PF4) or transforming growth factor beta (TGF-ß) (Rusciano, et al., BioEssays 14: 185-194 (1992)) may contribute to prolonged tumor cell survival and growth. Thus, locally-generated proteases my influence additional events following the secondary stabilized localization of tumor cells.

It is not known whether occupancy of TF by ligand (factor VII or VIIa) provides additional signals to the tumor cell that favors survival and proliferation.

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Binding of mAb TF8-5G9 to TF not only rapidly abolishes
function, but also decreases the binding of ligand
(Ruf, et al., <u>Thromb. Haemost. 66</u>: 529-533 (1991)).
The cytoplasmic domain of TF has been found to be
5   phosphorylated by a protein kinase C-dependent
mechanism, indicating that TF is associated with other
molecules involved in signal transduction (Zioncheck,
et al., <u>J. Biol. Chem. 267</u>: 3561-3564 (1992)). In
analogy to other members of the cytokine receptor
10   family (Fuh, et al., <u>Science 256</u>: 1677-1680 (1992)),
ligand-induced dimerization of TF may provide adaptive
or proliferative signals that support, or may even be
required for, the metastatic process. The findings
disclosed herein establish a requirement for ligand
15   binding and function of TF expressed on the tumor cell
surface. Specific functional inhibition of this
receptor molecule markedly reduces pulmonary
metastasis, providing evidence that locally generated
proteolytic activity at the site of tumor cell arrest
20   is a key event in metastatic implantation.

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<u>Example VIII</u>
<u>Functional Inhibition of TF and Metastasis:</u>
<u>Dosages and Inhibitory Mechanisms</u>

</div>

For the following experiments, the protocol
25   described in Example V above was followed. As noted
above, intravenous injection of M24met metastatic
melanoma cells into scid mice reliably produces a large
number of metastases in the lungs.

Tables 3A and 3B illustrate the effect of
30   functional inhibition of TF, in an experimental
pulmonary metastasis model. In the tables, the number
of metastases per mouse in each experiment is plotted
against the total dose of monoclonal antibody (or
buffer solution only) administered to each subject.
35   As illustrated in Table 3A below, a dose titration

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of TF8-5G9 demonstrates efficient inhibition of
metastasis at lower doses, which are all capable
inhibiting tissue factor function by more than 98%.
This suggests that antibodies capable of inhibiting
5   tissue factor function are useful inhibitors of
metastasis over a rather wide dosage range.

Table 3B depicts data from an experiment in which
antibody TF8-5G9 is compared with antibody TF9-6B4,
which inhibits tissue factor function via a different
10   means than TF8-5G9, through binding to a distinct
epitope in the amino-terminal module of tissue factor.
According to the data shown, TF9-6B4 inhibits
metastasis, though perhaps somewhat less potently than
TF8-5G9. This difference seems to be is consistent
15   with the reduced inhibitory potency of TF9-6B4 versus
TF8-5G9 observed in plasma.

Table 3A

FUNCTIONAL INHIBITION OF TF PREVENTS EXPERIMENTAL PULMONARY METASTASIS – ANTIBODY DOSAGE EXPERIMENTS –

Number of Cells Injected Into Each Mouse: 0.5×10^6 M24met

mAb (total dose)	No. Metastases/Mouse (total in both lungs)			
none	153	152	39	0
TF8-5G9 (1mg)	2	1	0	0
TF8-5G9 (0.5mg)	1	0	0	0
TF8-5G9 (0.1mg)	1	0	0	0
TF8-5G9 (0.05mg)	2	0	0	0

TABLE 3B

FUNCTIONAL INHIBITION OF TF PREVENTS EXPERIMENTAL PULMONARY METASTASIS – INHIBITORY MECHANISM –

Number of Cells Injected Into Each Mouse: 0.5×10^6 M24met

mAb (total dose)	No. Metastases/Mouse (total in both lungs)					
none	17	19	29	51	63	75
TF8-5G9 (1mg)	0	0	0	0	0	1
TF9-6B4 (1mg)	2	4	6	7	8	9

Example IX

Inhibition of Tumor-Associated TF In Vivo

1mg of mAb TF8-5G9 was injected into the tail
veins of scid mice and plasma was obtained from cardiac
5   puncture after four hours. The inhibition of TF
function by the mAb in the mouse plasma was determined
by preincubation of relipidated human TF with the
plasma for 30 minutes followed by determination of
residual TF function. A 1:2500 dilution of the mouse
10   plasma inhibited TF function by more than 90%. Thus,
specific functional inhibition by TF8-5G9 is not lost
in vivo.

In vivo inhibition of TF function in primary
tumors and metastases of the melanoma cell line M24met
15   was also analyzed, as follows. A cell suspension was
implanted subcutaneously leading to subcutaneous tumor
formation from which lymph node metastases developed.
Tumor tissue was isolated and stored frozen. After
mechanical disruption of the tumor integrity, TF
20   functional activity was established (see Fig. 5). TF
procoagulant activity was normalized on the basis of
the wet weight of tumor tissue or the M24met cell
pellet from culture in vitro and the quantitative
differences may relate to the relative amount of tumor
25   cells compared to host tissue.

Figure 5 illustrates the ability of anti-TF
monoclonal antibodies to inhibit tumor associated TF in
vivo and the TF expression of M24met melanoma cells in
vivo. Tissue factor procoagulant activity in mU/mg is
30   indicated on the vertical axis, whereas TF expression
in the presence or absence of mAb TF8-5G9 in cultured
cells, subcutaneous tumors, and lymph node metastases
is represented by the dark bars (- TF8-5G9) and light
bars (+ TF8-5G9) extending vertically from the
35   horizontal axis.

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Monoclonal antibody TF8-5G9 efficiently inhibited TF in both the subcutaneous tumor and the lymph node metastases. Residual procoagulant activity in the metastasis originates most likely from host-derived

5   activated macrophages which express TF during the immune response. Host TF is not, however, neutralized by TF8-5G9.

The foregoing specification, including the

10   specific embodiments and examples, is intended to be illustrative of the present invention and is not to be taken as limiting. Numerous other variations and modifications can be effected without departing from the true spirit and scope of the present invention.

15

WE CLAIM:

          1.   A therapeutic composition comprising, in unit dose form, immunoglobulin molecules capable of binding to and inhibiting the function of human tissue factor (huTF), in a pharmaceutically acceptable excipient.

          2.   The composition of claim 1, wherein said molecules comprise antibodies or immunologically active fragments thereof.

          3.   The composition of claim 2, wherein said antibodies are capable of immunoreacting with huTF locus I or locus II epitopes, or both.

          4.   The composition of claim 2, wherein said antibodies are monoclonal.

          5.   The composition of claim 4, wherein said monoclonal antibodies are selected from the group consisting of TF8-5G9 and TF9-6B4.

          6.   A method of making a medicament useful for treating or inhibiting metastasis, comprising admixing a therapeutically effective amount of immunoglobulin molecules capable of binding to and inhibiting the function of cell surface-expressed huTF with a pharmaceutically acceptable carrier or excipient.

          7.   The method of claim 6, wherein said immunoglobulin molecules comprise antibodies or immunologically active fragments thereof.

          8.   The method of claim 7, wherein said antibodies are monoclonal.

          9.   The method of claim 8, wherein said antibodies are selected from the group consisting of TF8-5G9 and TF9-6B4.

          10.   The method of claim 6, wherein said medicament is formulated for intravenous administration.

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11. The method of claim 6, wherein said therapeutically effective amount comprises an amount sufficient to reduce the cell surface expression of huTF by at least 20%.

5

12. The method of claim 6, wherein said therapeutically effective amount comprises an amount sufficient to produce plasma levels of said immunoglobulin molecules of about 0.05μg/ml to about 25μg/ml.

10

13. A method of identifying molecules capable of inhibiting metastasis, comprising:

(a) generating a suspension from a TF-containing source;

(b) incubating said suspension with a sample

15 of said molecules for a predetermined period of time, to form an admixture; and

(c) assaying said admixture for TF specific activity, thereby determining the ability of said molecules to inhibit metastasis.

20

14. A method of identifying compounds or molecules capable of inhibiting the expression of human tissue factor (huTF) on the cell surface, comprising:

(a) culturing tumor cells;

(b) admixing said tumor cells with a non-

25 cytotoxic test compound; ·

(c) incubating the resulting admixture for a predetermined period of time; and

(d) determining the amount of TF expressed per cell.

30

15. A method of inhibiting metastasis, comprising the administration of a therapeutically effective amount of immunoglobulin molecules capable of binding to and inhibiting the function of cell surface-expressed huTF.

35

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16.   The method of claim 15, wherein said molecules comprise antibodies or immunologically active fragments thereof.

17.   The method of claim 16, wherein said antibodies are monoclonal.

18.   The method of claim 17, wherein said antibodies are selected from the group consisting of TF8-5G9 and TF9-6B4.

19.   The method of claim 15, wherein said therapeutically effective amount comprises an amount sufficient to reduce the cell surface expression of huTF by at least 20%.

20.   A method of disrupting huTF·VIIa complex formation and function comprising the administration of an effective amount of antibodies or immunologically active fragments thereof, wherein said antibodies or fragments are capable of binding to cell surface-expressed huTF.
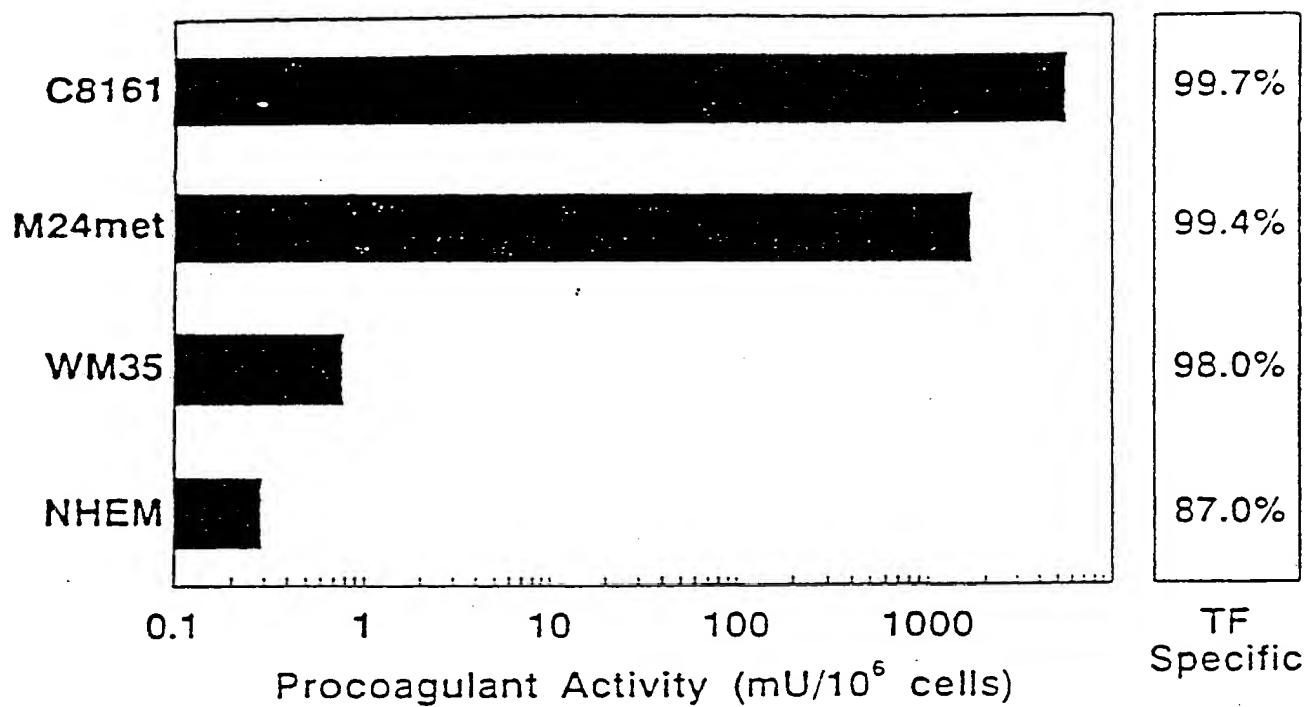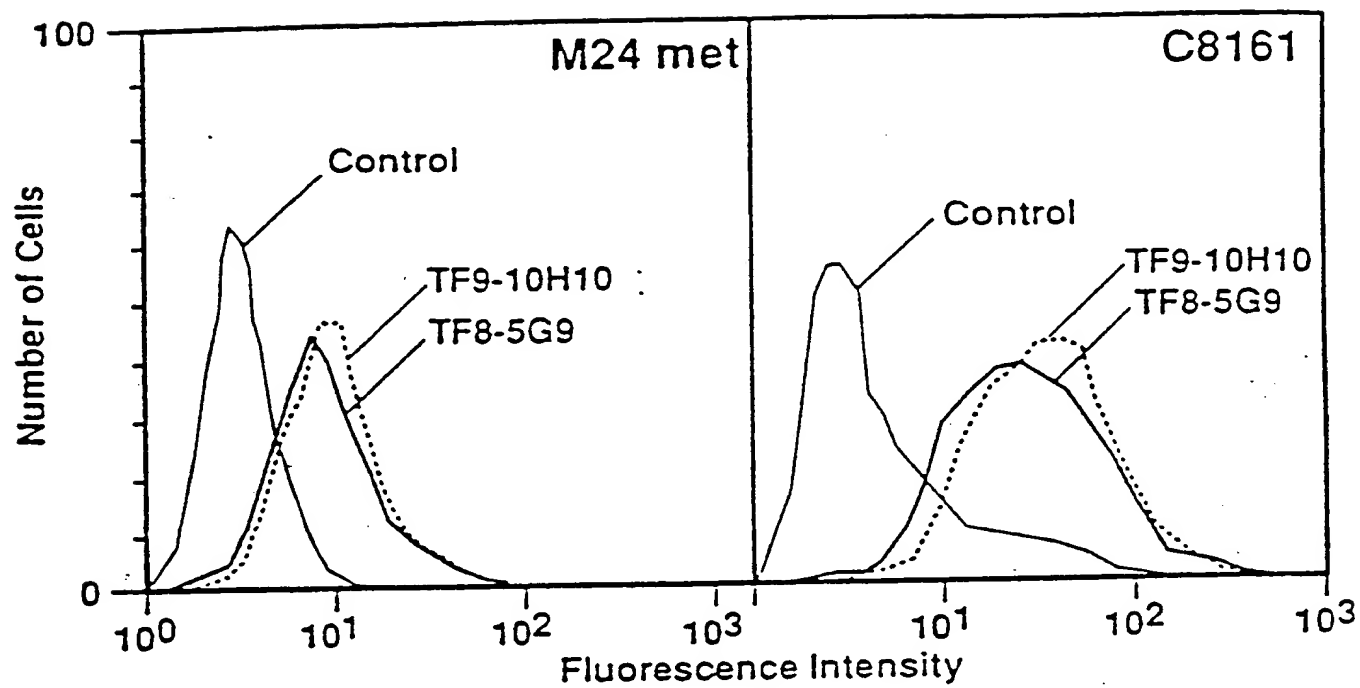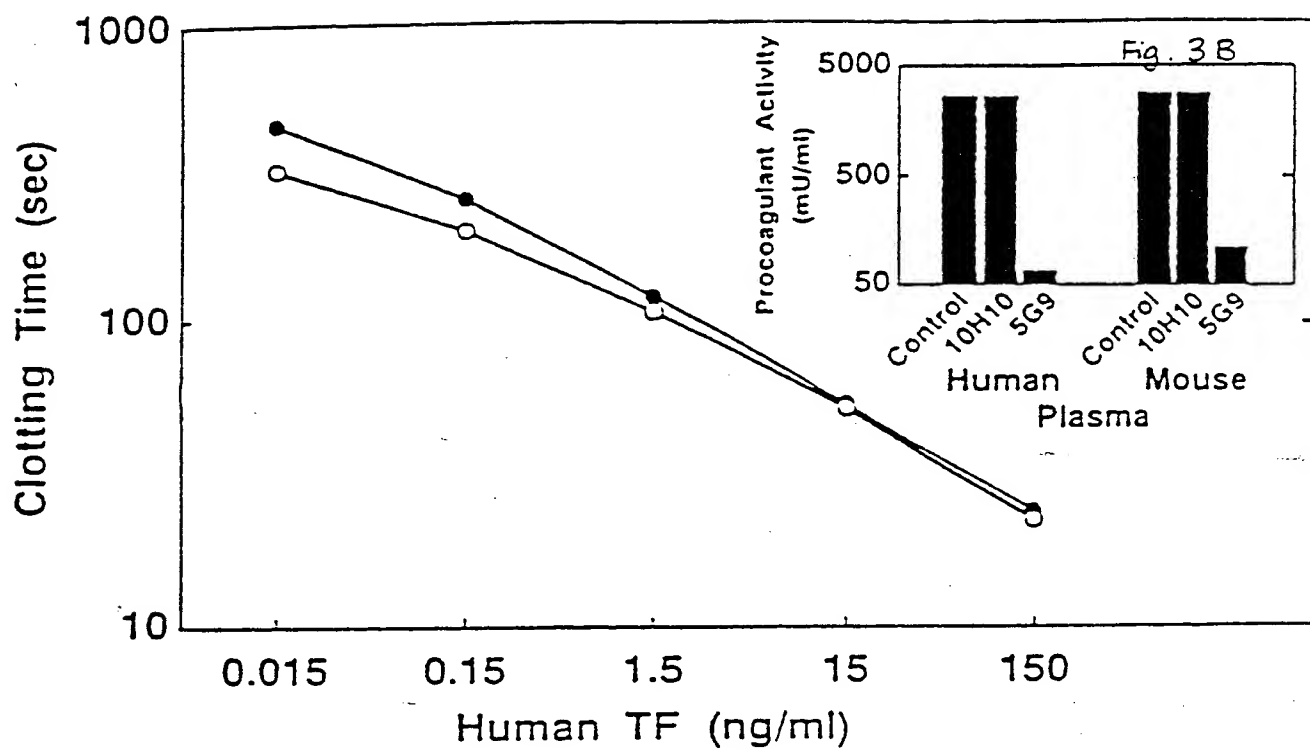
Fig. 1

Fig. 2A

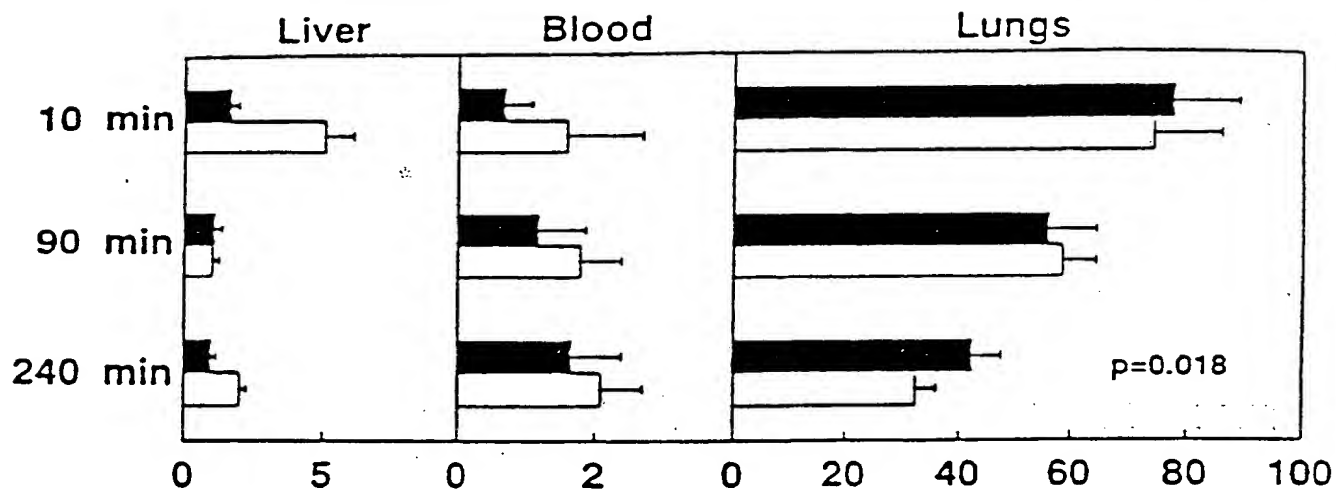Fig. 2B

Fig. 2

Fig. 3

Fig. 4A

Fig. 4B

Fig. 4
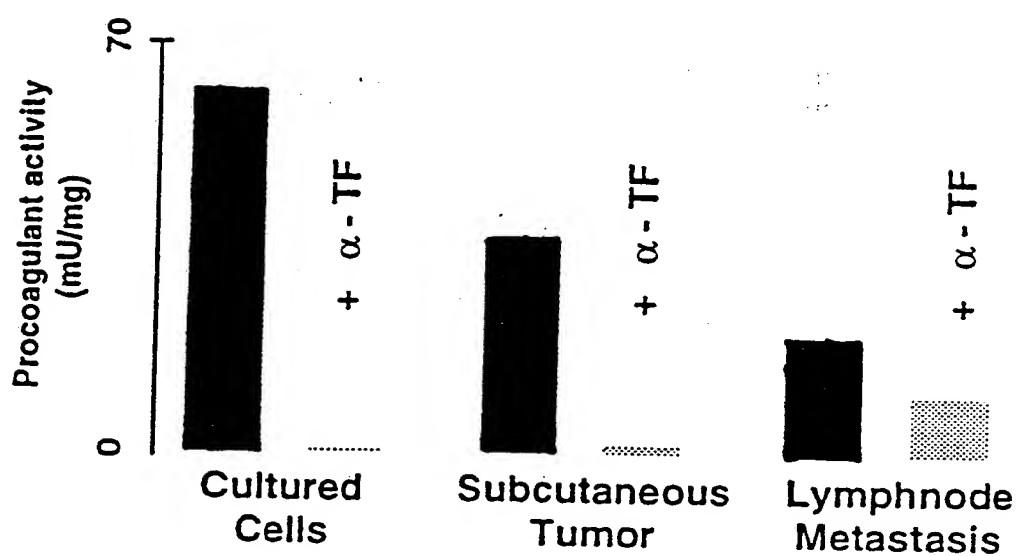
TF Expression of M24met
Melanoma Cell In Vivo

FIGURE 5

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A61K 39/395; G01N 33/53, 33/356
US CL   :424/85.8; 436/501, 536

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. :   424/85.8; 436/501, 536

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, BIOSIS, CA, MEDLINE, EMBASE, WPI

search terms: mueller, edgington, ruf, hutf, tissue factor, TF8-5G9, TF9-6B4, metastasis, tumor, cancer

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	TIBTECH, Volume 11, issued February 1993, W.J. Harris et al., "Therapeutic Antibodies - the Coming of Age", pages 42-45, see entire document.	1-12, 15-20
Y	US, A, 5,110,730 (Edgington et al.) 05 May 1992, see entire document.	1-20
Y,P	US, A 5,147,638 (Esmon et al.) 15 September 1992, see entire document.	1-20

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 October 1993	21 OCT 1993

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer PHILLIP GAMBEL
Facsimile No. NOT APPLICABLE	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/08047

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Thrombosis and Haemostasis, Volume 66, issued November 1991, W. Ruf et al., "An Anti-Tissue Factor Monoclonal Antibody Which Inhibits TF-VIIa Complex Is a Potent Anticoagulant in Plasma", pages 529-533, see entire document.	1-20
Y	Cancer Research, Volume 49, issued 01 October 1989, J. M. Silberberg et al., "Identification of Tissue Factor in Two Human Pancreatic Cancer Cell Lines", pages 5443-5447, see entire document.	1-20
Y	J. Cell. Biol., Volume 109, issued July 1989, T.A. Drake et al., "Functional Tissue Factor Is Entirely Cell Surface Expressed on Lipopolysaccharide-Stimulated Human Blood Monocytes and a Constitutively Tissue Factor-Producing Neoplastic Cell Line", pages 389-395, see entire document.	1-20
Y	Cancer Research, Volume 46, issued December 1986, M.B. Donati et al., "Cancer Procoagulant in Human Tumor Cells: Evidence from Melanoma Patients", pages 6471-6474, see entire document.	1-20
Y	Thrombosis Research, No. 3, Volume 52, issued December 1988, J.H. Morrissey et al., "Monoclonal Antibody Analysis of Purified and Cell-Associated Tissue Factor", pages 247-261, see entire document.	1-20
Y	Thrombosis Research, Volume 50, No. 4, issued May 1988, J.H. Morrissey et al., "Resolution of Monomeric and Heterodimeric Forms of Tissue Factor, The High-Affinity Cellular Receptor for Factor VII", pages 481-493, see entire document.	1-20

Form PCT/ISA/210 (continuation of second sheet)(July 1992)